UCLA
UCLA Electronic Theses and Dissertations

Title
Cyclin-D1 Modulation of Vitamin-D-Induced Gene Expression in Oral Keratinocytes

Permalink
https://escholarship.org/uc/item/7tq970cn

Author
Woods, Michael Connely

Publication Date
2013

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA

Los Angeles

Cyclin-D1 Modulation of Vitamin D-Induced Gene Expression in Oral Keratinocytes

A thesis submitted in partial satisfaction
of the requirements for the degree Master of Science
in Oral Biology by

Michael Connely Woods

2013

Abstract of Thesis

Cyclin-D1 Modulation of Vitamin D-Induced Gene Expression in Oral Keratinocytes

by

Michael Connely Woods

Masters of Science in Oral Biology

University of California, Los Angeles 2013

Professor Dr. Sanjay Mallya, Chair

Background: Oral cancer is the sixth most frequent cancer worldwide, with approximately 400,000 new cases diagnosed per year. More than 90% of oral cancers are characterized as squamous cell carcinomas (SCC), which develops in the cells of the oral epithelium, is a highly aggressive and destructive cancer with a propensity for invasion of surrounding tissues and recurrence. More than 50% of oral SCCs harbor amplification or overexpression of the cyclin D1 oncogene, a key regulator of the G1-S phase of the cell cycle. However, there is emerging evidence that cyclin D1 may have additional roles in transcriptional control. We recently discovered that cyclin D1 binds to the vitamin D receptor (VDR), a nuclear hormone receptor that mediates the actions of vitamin D.

Objectives: The objective of our research is to investigate the effect of cyclin D1 on vitamin D signaling. We will examine the effects of cyclin D1 on vitamin D-induced gene expression. We will also examine the effects of cyclin D1 overexpression on cellular localization of key components in both the vitamin D signaling pathway.

Methods: An oral keratinocyte cell line, engineered to overexpress cyclin D1 (OKF6-D1) will be used to examine the relationship between the VDR and Cyclin D1 pathways. To analyze global gene expression, we will use microarray analyses. Immuno-staining of key pathway components will be used to localize these components to nuclear or cytoplasmic compartments that can be visualized by immunofluorescence (IF) .

Results: Microarray analyses identified 599 known genes that were modulated by $1,25(OH)_2D_3$ in normal oral keratinocytes. These genes have known functions in keratinocyte differentiation, cell metabolism and immune modulation. Interestingly, cyclin D1 overexpression partially inhibited expression of several vitamin D-induced genes. The most significant effects were observed for keratinocyte differentiation genes. Vitamin D induced increase in VDR protein levels from 2 to 6 hours. VDR protein levels peaked at 6 hours for both OKF6 and OKF6-D1 keratincotyes. However, VDR protein levels were lower in OKF6-D1 keratinocytes at 12 and 24 hours of $1,25(OH)_2D_3$ treatment. Cyclin D1 did not alter $1,25(OH)_2D_3$- induced nuclear VDR translocation. Confocal immunofluorescence did not demonstrate any significant difference in the localization of VDR following $1,25(OH)_2D_3$ treatment. Interestingly, RXRα staining in $1,25(OH)_2D_3$ treated OKF6-D1 keratinocytes showed a small but noticeable reduction in peri-nuclear staining compared to OKF6 keratinocytes. Cyclin D1 overexpression markedly attenuated $1,25(OH)_2D_3$-induced VDR-RXR heterodimerization.

Conclusions: Cyclin D1 modulates $1,25(OH)_2D_3$-induced gene expression in oral keratinocytes, especially, genes involved in keratinocyte differentiation. Cyclin D1's effect on modulation of vitamin D signaling may be in part due to its inhibition of VDR-RXR heterodimerization.

The thesis of Michael Connely Woods is approved.

Shen Hu

Sotirios Tetradis

Sanjay Mallya, Committee Chair

University of California, Los Angeles

2013

Table of Contents

Abstract ....ii-iv

Introduction 1-6

Oral epithelium and keratinocyte development..1-2

Vitamin D 2

Vitamin D Receptor 3

Possible roles for vitamin D in prevention 3-4

Therapeutic interventions..3-4

Oral cancer risk factors 4

Biology of Oral Cancer 5

Oncogenes/ anti-oncogenes (Tumor suppressor genes)..5

Cell Cycle Regulatory; Cyclin D1 5

Link between VDR and cell cycle regulator 5-6

Materials & methods 7-9

Cells & culture conditions..7

RNA isolation & Microarray analysis 7

Real time PCR analysis 7

Immunoprecipitation & immunostaining..8

Immunofluoresence 9

Results 10-13

Microarray analysis 10

Real time-PCR analysis 11

VDR time course 12

Co-immunoprecipitation 12

Immunofluoresence..13

Discussion 14-16

Conclusion 17

Figures 18-27

Figure 1 18

Figure 2 19

Figure 3 20

Figure 4 21-22

Figure 5 23

Figure 6 23-24

Figure 7 24

Figure 8 25-27

References 28-32

Introduction

Oral cancer represents the sixth most frequent cancer, with nearly 400,000 new diagnoses each year [1]. The majority of oral cancers are classified as squamous cell carcinomas (SCC), which are characteristically aggressive, and locally destructive, often invading underlying bone and connective tissue. Thus, treatment demands aggressive resection to increase the likelihood of remission. Unfortunately, oral-pharyngeal cancer survivors have one of the highest susceptibilities to recurrence. Despite advancements in surgical techniques and medicine, survival rates have marginally improved over the past decades[2]. Consequently, there is an increasing need to identify preventative therapies for treating oral cancer survivors. Developing a strategy for prevention demands a comprehensive appreciation for the architecture that constitutes development of a healthy oral epithelium.

Oral Epithelium and Keratinocyte Development

The oral mucosa lining the intraoral compartment is divided into two major categories. First, the *masticatory mucosa* is comprised of a para-keratinized stratified squamous epithelium, which lines the dorsum of the tongue, hard palate, and the attached gingiva. Second, the lining mucosa is comprised of non-keratinized stratified squamous epithelium, which lines the remaining structures in the mouth. The epitheliums found in both mucosae constitute a stratified (layered) arrangement of keratinocytes. Within the epithelium keratinocytes are chronologically polarized, with immature cells localized at the base (Stratum basale) and differentiated cells at the surface as shown in **Figure 1.** Proliferating cells at the base of the epithelium provide a source of immature cells which gradually differentiate as they migrate away (towards the surface) to form a permeability barrier at the surface. Diverse cell to cell contacts, formed by proteins within the cellular membrane of these keratinocytes, +help to create a barrier and serve as markers for keratinocyte differentiation (involucrin, transglutaminase, etc). Calcium and vitamin D1,25$(OH)_2D_3$ are pivotal in regulating this process.

In vivo studies of the epithelium reveal a calcium gradient that increases from the base (stratum basale) to the surface, which supports in vitro findings. The gradient is responsible for providing a signal to migrating cells for differentiation. In addition to calcium, keratinocytes at the stratum basale are encouraged to differentiate by the

bioactive form of vitamin D (1,25$(OH)_2D_3$) via the nuclear vitamin D receptor (VDR). Interestingly, keratinocytes at the stratum basale possess the metabolic machinery needed for synthesis of the nuclear hormone 1,25$(OH)_2D_3$ (via the vitamin D-25 hydroxylase & 25 OHD-1OHase) from vitamin D3 in the same manner as liver and kidney enzymes[3-6].

Vitamin D: Vitamin D is a century old nutrient, initially used to prevent a devastating skeletal disease characterized by softening of the bone (Rickets). Today, we know a great deal more about the broad group of secosteroid's that constitute the vitamin and their involvement in mineral metabolism, skeletal integrity, and modulation of cell growth and differentiation in a variety of tissues. Since mammals possess a unique capacity to manufacture the nutrient when exposed to ultraviolet sun rays, vitamin D is not a true vitamin in the sense of the word1,25$(OH)_2D_3$, the bioactive form of the vitamin also known as calcitriol, is produced internally by sequential hydroxylation of the secosteroid precursor's obtained from the diet (ergocalciferol, D_2) or sun exposed skin (cholecalciferol, D_3)[7, 8]. The first hydroxylation is catalyzed by 25-hydroylase in the liver to produce 25-hydroxy vitamin D, the major circulating form. The final hydroxylation follows in the kidney's where 1-alpha-hydroxylase (1-αOHase) catalyzes the formation of 1,25$(OH)_2D_3$. A tightly controlled feedback system is directly exerted by the bioactive form of vitamin D at genes involved in the ligands production and degradation [9]. 1,25$(OH)_2D_3$suppresses renal expression of the 1-aOHase responsible for its synthesis, and induces expression of the 24-OHase responsible for its degradation to calcitroic acid[9, 10]. In addition1,25$(OH)_2D_3$auto-regulates the expression of its own receptor, VDR[11, 12] (**Figure 2**). Similarly, a classic endocrine system monitors serum levels of calcium, phosphorus, and PTH to maintain serum levels 1,25$(OH)_2D_3$[7, 8].

The biologic effects of 1,25$(OH)_2D_3$are orchestrated by the nuclear hormone vitamin D receptor (VDR). The interaction of 1,25$(OH)_2D_3$with VDR promotes its heterodimerization with the retinoid-x-receptor, a common partner of class II nuclear receptors[13]. The ligand activated heterodimer functions as a transcription factor for select target genes in the vitamin D pathway [14-16].

Vitamin D Receptor: Discovered in 1974, the vitamin D receptor (VDR) is a nuclear hormone receptor that facilitates differentiation by altering gene expression [17, 18]. Structurally VDR is composed of 3 distinct regions (FIGURE); N-terminal DNA binding domain, C-terminal ligand binding domain, and a linking region connecting the two domains [19, 20]. The binding domain contains a zinc finger typical of most steroid receptors[13, 21]. The ligand binding domain facilitates the formation of two independent surfaces for heterodimeric partner and co-regulatory complex interactions[22]. The role of ligand activated VDR is to direct cellular transcription by recruiting the appropriate machinery to specific genomic sites.

Upon ligand binding, the vitamin D receptor binds to specific DNA sequences known as VDRE's (vitamin D responsive elements). The VDRE's contain two hexameric nucleotide half sites, separated by 3 base pairs [14, 23]. The two half sites accommodate binding of a heterodimer comprised of the VDR/ RXR receptors[13]. Changes in gene expression are indirectly facilitated by VDRs recruitment of diverse co-regulatory complexes to these specific DNA sequences[24]. Co-regulatory complexes, include nucleosomal remodeling ATPases, enzymes with selective chromatin histone modifying capabilities, and RNA polymerase recruitment molecules.

Roles for Vitamin D in Prevention

Vitamin D's use in therapeutic interventions: A considerable amount of preclinical data suggest anti-cancer activities for vitamin metabolites ranging from anti-proliferation, anti-angiogenesis, pro-apoptosis, and pro-differentiation[25-27]. Clinical studies using vitamin D metabolites alone or in combination with chemotherapy have been conducted for various cancers, but mostly prostate cancer patients[28-38]. Single agent use of bioactive vitamin D (calcitriol) in preventive therapy produced limited success, with hypercalcemia representing a major drawback.

Interestingly, pre-clinical combination therapies suggest a synergistic or additive anti-cancer effect when calcitriol and cytotoxic agents are combined in chemotherapeutics[39-43]. Pre-clinical success with combination therapy has prompted the development of combination therapy trials. However, clinical trials have been underwhelming, largely due to confounded issues with dosing[44]. The first clinical studies for calcitriol were conducted in myelodysplastic syndrome (MDS) and acute leukemia (AML) patients[45-48]. The combination of cytarabine (chemotherapeutic agent) and calcitriol were successful in lowering remission of both disease in elderly patients, however the daily regimen was associated with hypercalcemia in 10-30% of patients. Critics of these experiments point to lack of dose optimization specific to anti-cancer properties, and emphasize the need to develop a maximum tolerated dose (MTD) or optimal biologic dose (OBD) (Vit D Cancer Review). Failed attempts at translating experimental findings into clinical success led to the development of vitamin D analogs in the hope of eliminating hyper-calcemia and enhancing anti-cancer properties[49, 50]. Although promising, the reduction in hypercalcemia afforded by vitamin D analogs has been plagued by reduced anti-cancer properties[25].

Risk Factors for Oral Cancer: Excessive environmental insults to the epidermis can be extremely detrimental over time. The rapidly proliferating cells at the base of the oral epithelium are of particular concern, since DNA damage caused by prolonged abuse provides an expedient pathway towards the unregulated growth characteristic of cancer.

Although numerous, the major risk factors for cancer of the oral cavity are known to be alcohol and tobacco consumption[51]. Other risk factors include poor oral hygiene; poor diet (low in fruits and vegetables) and low socioeconomic status[52, 53]. In addition to local carcinogen exposure, strong epidemiological and molecular evidence suggest a causal relationship between high risk human papilloma virus infection (HPV16/18) and oral SCC (8,9). More recently, two epidemiological studies have identified an association between vitamin D deficiency and oral squamous cell carcinoma[54, 55].

The Biology of Oral Cancer

Oncogenes/ anti-oncogenes (tumor suppressor genes): Cancer results from a multi-step process of gene activation or inactivation. The discovery of consistently amplified genes (cellular oncogenes) in specific cancers provided an inclination of their role in the pathogenesis of cancer[56-59]. Oncogenes, represent normal genes with the potential to cause cancer when highly expressed. Activated oncogenes allow cells to bypass cell death (apoptosis) in favor of proliferation and survival. There are several mechanisms responsible for their overexpression including; gene mutation [60], chromosomal duplication[61], and chromosomal translocation[62, 63]. Certain neoplasms frequently amplify such genes (N myc in neuroblastoma[64-66], L myc in small cell lung cancer [61, 67, 68]) of particular interest in our research is cyclin D1 in oral cancer, which has been shown to be amplified in more than 50% of OSCCs[69, 70].

Cell cycle regulator: cyclin D1: Cyclin D1 is a key regulator of the G1-S phase, which precedes cell cycle entry and proliferation. It binds to and activates cyclin-dependent kinases (CDK4 and CDK6), which encourage cell cycle progression by inactivating the retinoblastoma protein, a well-known tumor suppressor (**Figure 3**). This pathway is generally accepted as the central source of cyclin D1's oncogenic function. Thus, cyclin D1's amplification represents a likely driver of unregulated proliferation. Not surprisingly, the amplification of cyclin D1 is common in oral SCC [69-73]. Unclear, is whether cyclin D1's role in pathogenesis is dependent on its kinase partners.

Link between VDR and Cell Cycle Regulator

Previous research in our laboratory investigating the impact of over-expressing CDK4/6, revealed an inability to sufficiently disrupt keratinocyte differentiation in a manner that simulated keratinocytes in oral OSCC [74]). These findings suggest that cyclin D1's role in oncogenesis may be mediated through some other means. The precise mechanism by which cyclin D1 exerts its oncogenic effect remains unclear. In previous studies, we have used a transgenic animal model to demonstrate that cyclin D1 overexpression enhances susceptibility to carcinogen-induced oro-esophageal neoplasia [75]. Emerging evidence

suggests that in addition to its regulation of the cell cycle, cyclin D1 may plays a part in transcription. Studies from numerous cell types have revealed that cyclin D1 binds transcriptional regulators including the estrogen receptor, androgen receptor, thyroid receptor, and STAT3[76, 77]. These studies helped shape our hypothesis, that cyclin D1 has a role in transcriptional control, and that this novel interaction contributes to its oncogenic function[76]. In support of this hypothesis, previous work in our lab demonstrates that cyclin D1 associates with the receptor responsible for directly mediating vitamin D's signaling effects (VDR). We have recently found that cyclin D1 modulates the transcriptional activity VDR, thereby altering the sensitivity of oral keratinocytes to vitamin D.

In this study we aim investigate the impact of cylin D1 over-expression on vitamin D signaling. Afffymetrix was used investigate the functional significance of the cyclin D1-VDR association on vitamin D induced gene expression. Microarray findings were validated with RT-PCR analysis. Secondly, we aim to investigate the impact of cyclin D1 over-expression on the localization and interaction of Cyclin D1 and components critical to vitamin D signaling (VDR & RXR). Both immunofluorescence and co-immuno-precipitation assays (data not shown) were used to investigate changes in the localization vitamin D signaling components and VDR-RXR hetero-dimerization respectively.

Materials & Methods

Cells & culture conditions

OKF6-TERT1 cells (obtained from Dr. James Rheinwald, Brigham and Women's Hospital, Boston, MA) are immortalized, but non-transformed oral keratinocyte cell lines that stably express hTERT—the catalytic subunit of telomerase [78]. Cells were grown in keratinocyte serum-free medium (Invitrogen corporation, Carlsbad, CA) supplemented with bovine pituitary extract (25 µg/ml), epidermal growth factor (0.2 ng/ml), penicillin (100 IU/ml) and streptomycin (100µg/ml). The calcium concentration of the media was adjusted to 0.03mM. To induce keratinocyte differentiation, cells were grown either in the presence of 1,25$(OH)_2D_3$ (10^{-7}M). OKF6-D1 cells were derived from OKF6-TERT1 cells by stable transduction with lentiviral constructs expressing the human cyclin D1 cDNA as previously described [74].

RNA isolation

For RNA isolation, total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) and partially purified using the RNEasy Micro kit[79]. The aqueous phase from Trizol lysis was used as the starting material in the column based purification. DNase was used following on column purification to eliminate any remaining trace elements of DNA. The RNA concentration was determined using by spectrophotometery. The microarray was performed on the Affymetrix U-133 +2.0 platform.

Quantitative real-time PCR

For quantitative PCR analyses, total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed using random hexamers. The cDNA templates were amplified using gene-specific primers to amplify S100A7, S100A9, SPRR1A, SPRR1B, SerpinB3 and the TATA-binding protein (TBP, as endogenous control). Sequences of the primers are as follows: S100A7 (Forward: 5'-TCTTGTCATCACGTCTGGTGT-3', Reverse: 5'- CTACTCGTGACGCTTCCCA-3'), S100A9 (Forward: 5'-TCAGCTGCTTGTCTGCATTT-3', Reverse: 5'-GGAATTCAAAGAGCTGGTGC-3'), SPRR1A (Forward: 5'-

TCACCTGCTGCTGCTGAG-3’, Reverse: 5’-AGCCCATTCTGCTCCGTAT-3’); SPRR1B (Forward: 5’- AGGGCTGCTTCTGCTGC-3’, Reverse: 5’-GCAGTTCTAAGGGACCATACAGA-3’); SerpinB3 (Forward: 5’-AGAGCTTGTTGGCGATCTTC-3’; Reverse: 5’- AACACCACAGGAAAAGCTGC-3’); Cyclin D1 (Forward: 5’-CAGAGGCGGAGGAGAACAAA-3’, Reverse: 5’-ATGGAGGGCGGATTGGAA-3’); TBP (Forward: 5’-CACGAACCACGGCACTGATT-3’, Reverse: 5’-TTTTCTTGCTGCCAGTCTGGAC-3’). Real time PCR reactions were done in triplicate on an ABI 7900HT real-time PCR unit using the SYBR green detection and analyzed using the delta-delta CT method.

Micorarray Analysis

Human U133plus2.0 array hybridizations were performed by UCLA Clinical Microarray Core following standard Affymetrix GeneChip Expression Analysis protocol. The acquisition of array image was undertaken by using Affymetrix GeneChip Command Console 1.1 (AGCC). Subsequent raw data were analyzed using Partek genomics Suite 6.4. We used RMA algorithms for data normalization. Thresholds for selecting significant genes were set at >= 2-fold and $p<0.05$. Genes met both criteria simultaneously were considered as significant changes. Global functional analyses, network analyses and canonical pathway analyses were performed using Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com).

Co-immunoprecipitation & Western blot analyses

Cells were treated with $1,25(OH)_2D_3$ 6 hours prior to lysis in all experiments except VDR time course. Vitamin D treatment in VDR time course varied from 0,2,6,and 12 hours. Following incubation with VDR cells were lysed in preparation for immunstaining.

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer for 1 hour on ice. Cell debris was removed by centrifugation at 10,000xg for 10 minutes at 4° C. For immunoblotting, equal amounts of protein were layered onto reducing SDS-PAGE gels and electroblotted onto PVDF membranes. For immunoprecipitation, pre-cleared whole cell lysates, containing approximately 0.8 to 1 mg of total protein, were incubated with anti-VDR (Clone 9A7, Affinity Bioreagents, Golden, CO, or

clone D-6, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-cyclin D1 (Clone DCS-6, Santa Cruz Biotechnologies, Santa Cruz, CA) antibodies for 12-16 hours. Pre-equilibrated protein G–agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) were added and collected by centrifugation after 2 h of incubation and then gently washed three times with the lysis buffer. Bound proteins were eluted by heating in lamelli buffer, resolved on an SDS–PAGE gel and transferred onto PVDF membranes.

The following primary antibodies were used for immunoblotting: mouse monoclonal anti-human cyclin D1 (clone DCS-6), rat monoclonal anti-chicken VDR (Clone 9A7), mouse monoclonal anti-human VDR (Clone D-6), mouse monoclonal anti-human involucrin (clone SY-5, Sigma-Aldrich, St. Louis, MO), rabbit polyclonal anti-human RXR (ΔN197 and D-20, Santacruz Biotechnology) and mouse monoclonal antibody against human GAPDH, as loading control (Abcam Inc, Cambridge, MA). Binding of the primary antibody was detected using the appropriate horseradish peroxidase-conjugated IgG and Luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunofluoresence

Cells were allowed to attach overnight to chambered glass coverslips. The calcium concentration was unchanged for OKF6 keratinocytesor replaced with 1,25 vitamin D (10^{-7} M) and the cells were incubated for 6 hours. Cells were fixed with 4% formaldehyde for 10 minutes and washed in PBS. To detect vitamin D signaling components (VDR, RXRα) or cyclin D1, cells were blocked with 5% goat serum and incubated with the primary antibody (mouse monoclonal anti-human VDR (D6), rabbit polyclonal anti-human RXRα (D20), and mouse monoclonal anti-human Cyclin D1 (DCS6) from Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours at room temperature. Bound primary antibody was detected using an Alexa Fluor 568-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Invitrogen Corporation, Carlsabad, CA) and the nuclei were counterstained with DAPI (Invitrogen Corporation, Carlsabad, CA). Sections were examined by confocal microscopy.

Results

Microarray Data

Microarray analysis was used to investigate the global impact of cyclin D1 over-expression on vitamin D signaling. We used a stringent threshold for differential gene expression (± 3-fold change over untreated, baseline expression). Only the highest differentially expressed genes were selected for further investigation. We identified 599 known genes that were modulated by $1,25(OH)_2D_3$ in normal oral keratinocytes these genes have known functions in keratinocyte differentiation, cell metabolism and immune modulation (**Figure 4**)

Heat Mapping: Microarray expression profiling demonstrates differences in gene expression for both $1,25(OH)_2D_3$ treatment and cyclin D1 over-expression. A gene was accepted as differentially expressed if there was a three-fold or greater difference in its expression between study groups. This segregated 770 differentially expressed genes. The heat map revealed a distinctive pattern in the keratinocytes treated with $1,25(OH)_2D_3$, and a less distinctive pattern for keratinocytes over-expressing cyclin D1. Genes that showed increased expression are shown in red and genes showing decreased expression in blue. Clearly, $1,25(OH)_2D_3$ treatment had a significant impact on the expression profiles of both OKF6and OKF6-D1 over-expressing keratinocytes. (**Figure 4A**).

PCA Mapping: To better visualize the large data sets obtained from the DNA expression microarray studies, principle component analysis (PCA) was performed. PCA uses a three-dimensional scatter plot, derived from the gene list used to generate the heat map. This analysis uses distance between samples to illustrate their unique differences. The y-axis indicates that 27% of the variability between groups was attributed to the over-expression of cyclin D1. The x-axis indicates that 48% of the variability between groups could be attributed to $1,25(OH)_2D_3$ treatment. Thus, $1,25(OH)_2D_3$ treatment is responsible for the majority of variability in gene expression. The impact of $1,25(OH)_2D_3$ treatment on the expression profile can be visualized by observing horizontal distance between proliferating keratinocytes (OKF6/ OKF6-D1) and keratinocytes induced to differentiate (OKF6 + vit D/ OKF6-

D1 + vit D respectively) with 1,25$(OH)_2D_3$. The horizontal shift between these respective groups suggests that the expression profile of differentiating keratinocytes differs considerably from proliferating keratinocytes irrespective of the over-expression of cyclin D1. Interestingly, the PCA map clearly indicates that the difference in gene expression profiles between OKF6and OKF6-D1 over-expressing keratinoctyes (indicated by the vertical distance between E & D1 on PCA map), becomes smaller following 1,25$(OH)_2D_3$ treatment (indicated by vertical difference between E+vit D & D1+vit D) (**Figure 4B**). This finding suggests that vitamin D treatment brings the expression profile of OKF6-D1keratinocytes closer to that of OKF6 keratinocytes (and vice versa), thus reducing the extent of variability introduced by cyclin D1 over-expression.

RT-PCR Analyses

A group of genes with functions related to cornified envelope development (S100A7, S100A9, SPRR1A, SPRR1B, and SerpinB3) were among those found to be highly differentially expressed. RT-PCR was used to validate the differential expression of these candidate genes in OKF6and OKF6-D1 over-expressing KCs treated with vitamin D. In each experiment the expression level in OKF6cells were used as a baseline for comparison. Vitamin D treatment in OKF6and OKF6-D1 cells induced increased expression of the majority of these genes. However, the scale of induction was reduced in keratinocytes over-expressing cyclin D1 (**Figure 5**). These findings suggest that cyclin D1 over-expression is associated with a deregulation of vitamin D signaling. We hypothesized that mechanism for such deregulation is either directly attributed to cyclin D1's interaction with VDR or indirectly related to cyclin D1 induced changes in the localization of vitamin D signaling components. To investigate our hypotheses further we implemented co-immuno-precipitation assays and immunofluorescence respectively.

VDR Expression Time Course

To investigate Cyclin D1's role in deregulation of vitamin D signaling, we monitored changes in VDR expression following ligand treatment. Western blot analysis of lysates from OKF6and OKF6-D1 over-expressing keratinoctyes treated with 1,25(OH)$_2$D$_3$ for 2,6,12,and 24 hours were used to examine changes in VDR induction. Expression of the VDR protein is modulated by 1,25(OH)$_2$D$_3$–treatment. In OKF6 keratinocytes VDR expression by increased by 2 hours and was maximal after 6 hours of treatment, after which a decrease in the amount of VDR protein was detected (**Figure 6**). A similar patter was also observed in cyclin D1 cells. However, VDR protein levels following 12 and 24 hours of 1,25(OH)$_2$D$_3$–treatment, was considerably lower in cyclin D1 over-expressing cells compared to OKF6 cells (**Figure 6**). These findings suggest that cyclin D1 may modulate vitamin D signaling by modulating ligand-induced VDR expression.

Co-Immunoprecipitation Assays

VDR's transcriptional activity is regulated by heterodimerization with RXR. To further examine the functional significance of VDR-cyclin D1 association, we examined whether cyclin D1 overexpression modulated VDR–RXR association, as determined by co-immunoprecipitation assays. In the first set of experiments we used an anti- RXR antibody (ΔN197) that recognizes all three isoforms of RXR. Using this antibody, RXR–VDR association was detected in untreated OKF6and cyclin D1 overexpressing cells. However, there was a marked attenuation of RXR–VDR association in 1,25(OH)$_2$D$_3$–treated cyclin D1 overexpressing cells (**Figure 7A**). In the second set of experiments we used an antibody that specifically detects the RXRα isoform. There was no detectable RXR–VDR association in either OKF6or OKF6-D1 overexpressing cells. In OKF6cells, RXRα–VDR association was induced by 1,25(OH)$_2$D$_3$-treatment. In contrast, there was almost complete attenuation of RXRα-VDR association in 1,25(OH)$_2$D$_3$-treated cyclin D1-overexpressing cells (**Figure 7B**). Overall, these data demonstrate that cyclin D1 may interfere with VDR-RXR heterodimerization, specifically the RXRα–isoform.

Immunofluorescence

To investigate whether an abrogation of VDR–RXR heterodimerization was due to a direct inhibition of the binding of the two proteins, or due to cyclin D1-induced cellular sequestration of the two proteins, we used immunofluorescence to examine cellular localization of VDR and RXR. OKF6 and OKF6-D1 cells were treated with $1,25(OH)_2D_3$ for 6 hours. Cellular localization of VDR, RXR and cyclin D1 was examined by immunofluorescence. Cells were stained with DAPI to counterstain the nucleus. (**Figure 8**).

Cyclin D1: Immunofluoresence (IF) demonstrated predominantly nuclear localization of cyclin D1 in untreated OKF6 cells. $1,25(OH)_2D_3$ treatment of OKF6 cells did not alter the pattern of localization. In contrast, untreated OKF6-D1 cells displayed robust cytoplasmic and nuclear staining. Similar to OKF6 cells, vitamin D treatment had a minimal effect on cyclin D1's localization. (**Figure 8A**).

VDR: Immunofluorescence demonstrated VDR localization in both cytoplasmic and nuclear, with predominant nuclear accumulation in untreated OKF6 cells. Upon $1,25(OH)_2D_3$-treatment, VDR localization was predominantly nuclear. This pattern of localization was similar in OKF6-D1 cells. Interestingly, the intensity of VDR staining was lower in OKF6-D1 keratinocytes compared with OKF6 cells (**Figure 8B**).

RXRalpha: In untreated OKF6 cells RXRalpha was detected in both the nucelau and the cytoplasm, although the pattern of staining was diffuse in both compartments. Upon $1,25(OH)_2D_3$-treatment, RXR localized was changed to a strong peri-nuclear accumulation. A similar pattern of RXR localization was detected in OKF6-D1 cells. However, $1,25(OH)_2D_3$ –treated OKF6-D1 cells showed a more diffue perinuclear accumulation compared with OKF6 cells (**Figure 8C**).

Discussion

Cyclin D1 amplification is a frequent aberration that contributes to OSCC pathogenesis. However the mechanism of its pathogenicity is not fully understood. Cyclin D1 is frequently amplified in cancer, leading to unregulated proliferation and a diminished ability to terminally differentiate. Cyclin D1's oncogenic capacity is attributed to alterations in its role as "gate keeper" to cell cycle entry. The mitogenic effects of cyclin D1 are generally attributed to its two cyclin dependent kinase (CDK) partners, CDK4 and CDK6. This pathway has been thought to be the primary mechanism for cyclin D1's oncogenic functions.

Interestingly however, our discovery of an interaction between cyclin D1 and the nuclear hormone, VDR, raises questions about additional pathways for cyclin D1s pathogenesis. Although new to oral keratinocytes, the link between cyclin D1 and transcriptional machinery (specifically nuclear hormone receptors) is not uncommon. Previous studies have demonstrated from other cells types has shown that cyclin D1 interacts with other transcriptional regulators—the estrogen receptor in mammary epithelial cells, and the androgen receptor in prostate cells ([80, 81]). The intersection of the vitamin D and cyclin D1 pathway is intriguing because it links two essential hallmarks of cancer: deregulation of proliferation and differenation. Vitamin D's less commonly known role in cell cycle inhibition and the promotion differentiation, consists of a cascade of intracellular interactions that lead to the modulation of gene expression, whereas cyclin D1 promotes cell proliferation.

Our microarray results clearly indicate a difference in $1,25(OH)_2D_3$–induced gene expression between cyclin D1-overexpressing and control oral keratinocytes. In particular, is cyclin D1's effect on modulation of $1,25(OH)_2D_3$–induced expression of keratinocyte differentiation genes. Our data strongly support a role for cyclin D1 in modulating vitamin D signaling in oral keratinocytes. Interstingly, we previously showed that cells over-expressing either cyclin D1 or CDK4/6 retain the ability to respond to calcium-induced differentiation ([74]). Thus, our current finding of cyclin D1' effect on deregulated vitamin D-signaling suggest that these effects are specific to the vitamin D-signaling pathway and are not non-specific effects related to an increased proliferative state induced by cyclin D1 overexpression.

In considering the mechanism for cyclin D1's effects on vitamin D signaling, we speculated that cyclin D1 overexpression may alter cellular localization of VDR and/or it's partner RXR. Our data did not indicate any major alteration in cellular trafficking of these two proteins in cyclin D1-overexpressing cells. However, we detected that cyclin D1 markedly inhibited RXRα –VDR association (**Figure 7**). Furthermore, VDR protein levels were considerably lower in cyclin D1 over-expressing keratinocytes (**Figure 6**). The loss of this interaction has direct implications for the transcription of vitamin D genes. Interestingly, ligand induced changes in the VDR protein revealed diminished protein levels after 6 hours of treatment in D1 over-expressing cells; however VDR protein levels remained elevated in OKF6cells 24 hours after exposure to vitamin D (**Figure 6**). Reduced levels of VDR would directly impact the vitamin D pathways ability to stimulate the expression of genes in the vitamin D pathway. The mechanism of deregulated vitamin D signaling could be explained in a number of ways, including changes in the localization of key signaling components or key signaling interactions. We conducted immunofluorescence to rule out changes in the localization of key components as a potential mechanism for disruption. The lack of change in the localization of key vitamin D signaling components (RXR & VDR) and cyclin D1 as seen in immunofluorescence ruled this out as a potential explanation. Although no major changes in localization were observed in vitamin D treated keratinocytes overexpressing cyclin D1, notable minor changes were observed. Peri-nuclear localization of RXRα was observed in both ligand treated OKF6and OKF6-D1keratinocytes, suggesting that the VDR-RXRα interaction occurs somewhere along the nuclear membrane (**Figure 5C**). This finding is consistent with the notion that RXR and VDR form a complex that targets the nucleus for modulation of gene expression. We did observe reduced peri-nuclear staining and a shift towards a more diffuse cytoplasmic staining in ligand treated D1 cells. This finding was consistent with our co-immunoprecipitation results, which show inhibition of the RXRα – VDR interaction in ligand treated D1 cells. Disruption of this complex will inhibit VDRs ability to modulate gene expression. The nuclear accumulation of VDR following ligand treatment was observed in both OKF6and OKF6-D1 cells. This finding ruled out nuclear exclusion of VDR as a plausible means of deregulating vitamin D signaling.

In summary, our results highlight the importance of the interaction between the cyclin D1 oncogene and the vitamin D pathway. We have shown that the overexpression of cyclin D1

is associated with disruption of the normal physiology attributed to vitamin D signaling and inhibition of early signaling interactions. The early interaction of VDR and RXR are required for modulation of gene expression. The vitamin D signaling pathway is important for normal keratinocyte physiology (pro-differentiation and anti-proliferation), especially since it has been shown too important in other types of cancer. This is the first study linking vitamin D signaling to the specific oncogenic interactions of cyclin D1. In the future we hope to further explore the implications of cyclin D1-VDR interaction by examining the impact on VDRs ability to bind DNA using chromatin immunoprecipitation (ChIP) assays to quantify changes in VDR's ability to bind DNA.

Conclusion

In conclusion, we demonstrated that the over-expression of cyclin D1 in OKF6 cells leads to the deregulation of vitamin D induced gene expression. The loss of vitamin D signaling is evidence by partial inhibition of vitamin D induced genes, reduction in VDR protein levels, and disruption of VDR-RXR interactions.

Figures

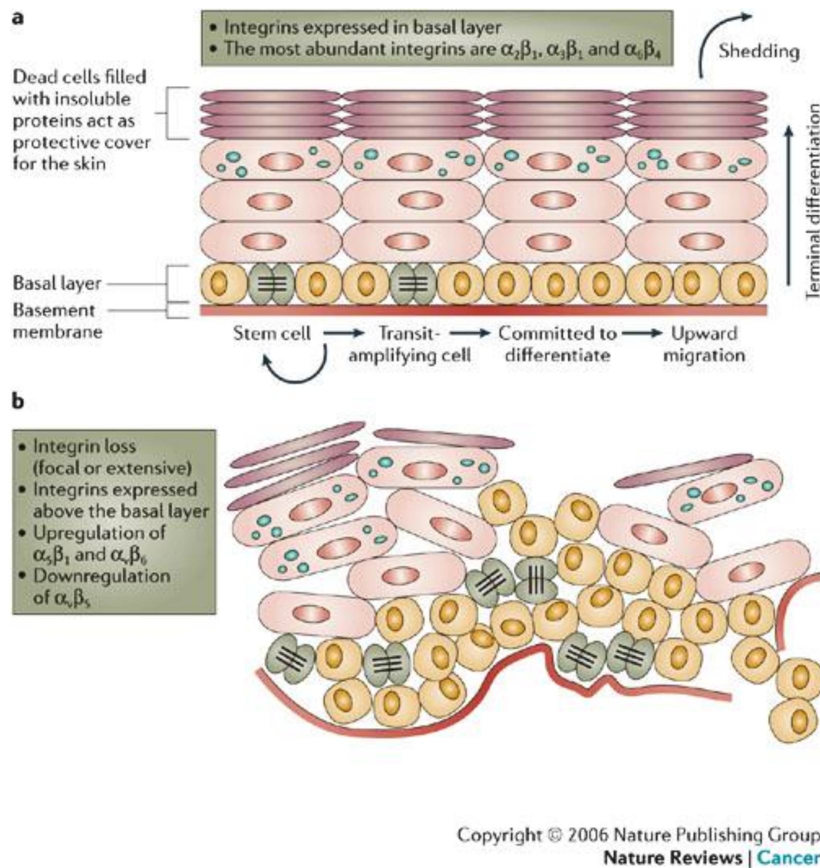


Figure 1. **Cellular organization in normal epidermis and squamous-cell carcinoma.** (a) In the human epidermis and oral epithelium immature proliferating keratinocytes are derived from stem cells at the basement membrane. Keratinocytes that destined for terminal differentiation, loose their ability to divide and detach from the basement membrane. The migration of these cells from the basal layer correlates the formation of tight intercellular interactions as these cells become specialized to form the protective outer covering of the skin. (b) Squamous cell carcinomas are characterized by focal or extensive loss of the basement membrane, increased proliferating cells, and reduced number of cells undergoing terminal differentiation. The spatial organization of the epithelium is disrupted, such that cells no longer maintain discrete arrangement of layers that constitutes the protective network of cells found in normal epithelium. Reference: Sam M. Janes and Fiona M. Watt. (2006). New roles for integrins in squamous-cell carcinoma. *Nature Reviews Cancer*. doi:10.1038/nrc1817

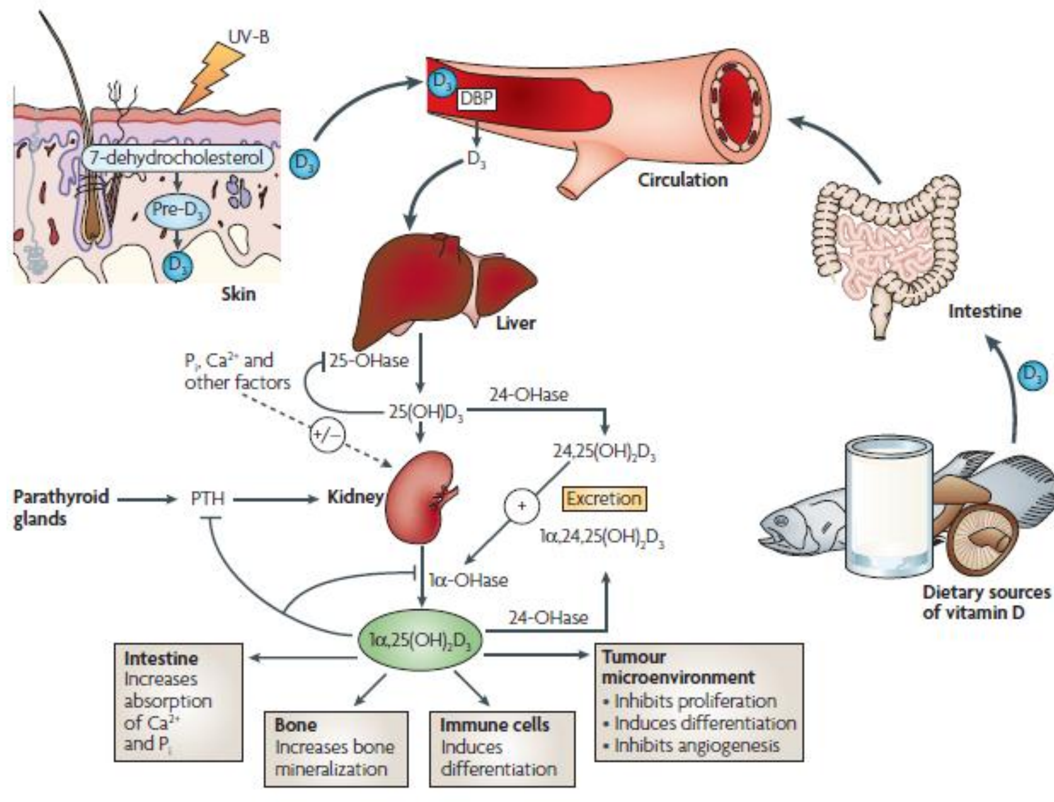


Figure 2. **Vitamin D metabolism.** Vitamin D is a broad term used to describe a number of derivatives. The primary sources of vitamin D are sun exposed skin and the diet. In the skin, cholesterol (7-dehydrocholesterol) is converted to pre-vitamin D-3 in response to ultraviolet B rays from the sun. Pre-vitamin D3 absorbed from the diet or synthesized in the skin binds to vitamin D-binding protein (DBP) in the bloodstream for transport to the liver. In the liver D3 is hydoxylated by 25-hydroxylases to the major circulating from 25-hydroxy D3. Stimulated by parathyroid hormone and suppressed by Ca2+, Pi and 1α,25(OH)2D3 itself, the kidney converts this secosteroid (25 OHD3) into the bioactive hormone 1α,25(OH)2D3 (calcitriol) from 25(OH)D3. This hormone has numerous effects on various target tissue(highlighted above). Deeb, K.K., D.L. Trump, and C.S. Johnson, *Vitamin D signalling pathways in cancer: potential for anticancer therapeutics.* Nat Rev Cancer, 2007. **7**(9): p. 684-700.

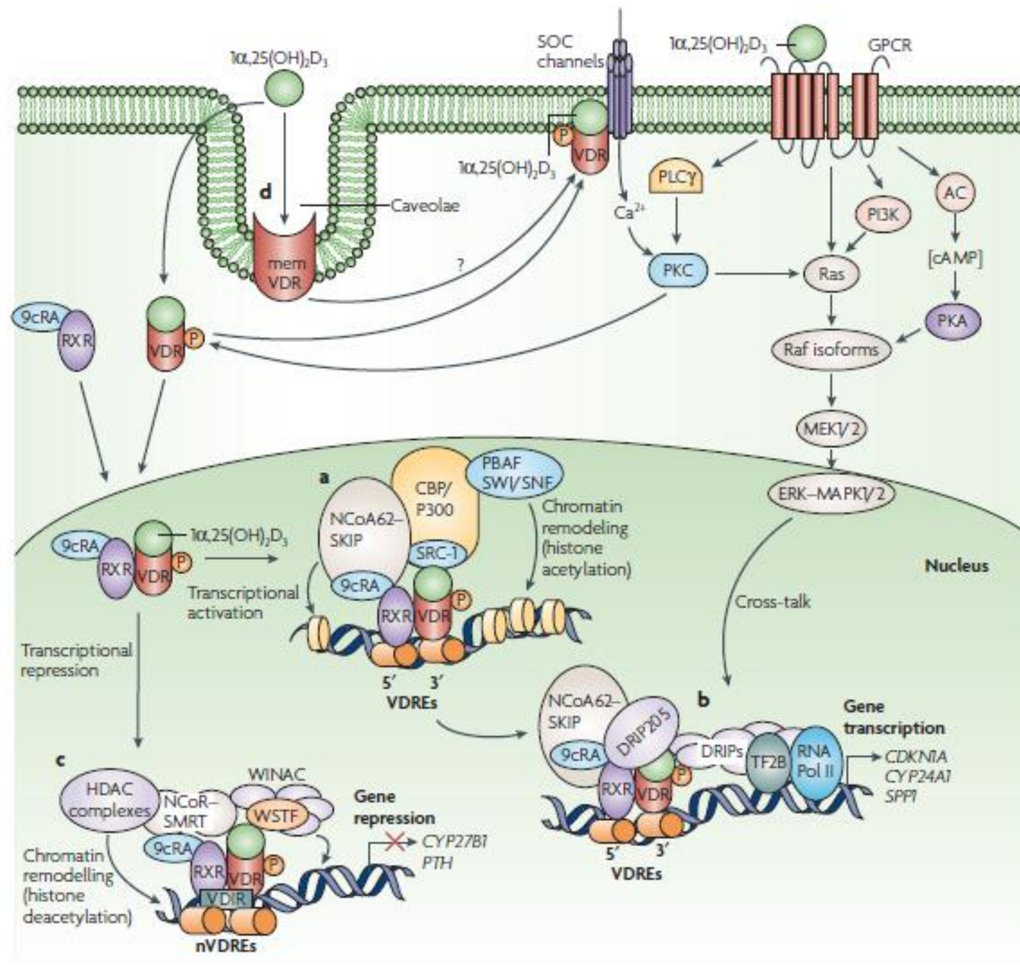
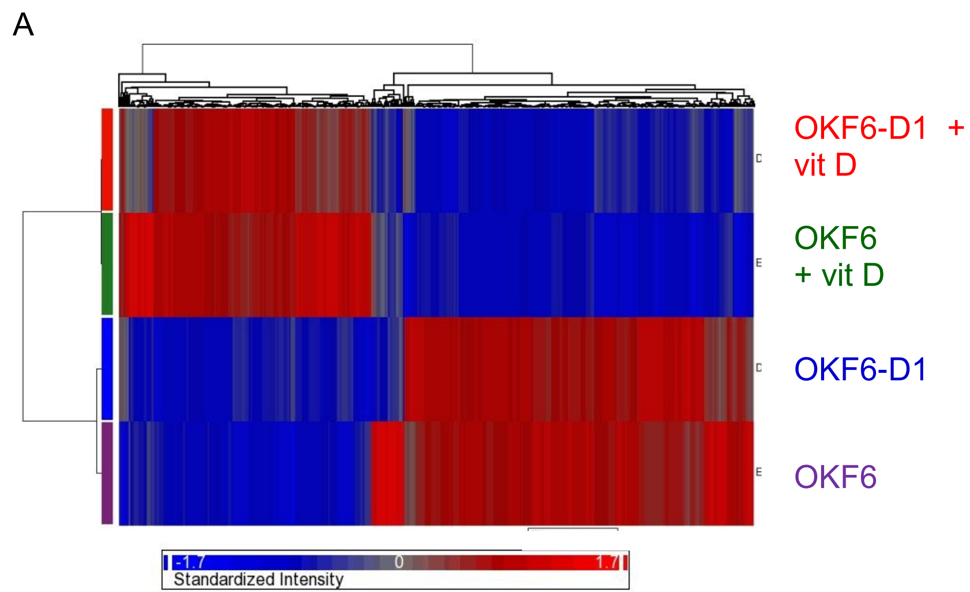


Figure 3. **1α,25(OH)2D3-mediated transcriptional regulation.** The classical actions of bioactive vitamin D3 are mediated by the vitamin D receptor (VDR), and 9-cis-retinoic acid receptor (RXR) which complex to form an active transcription factor. Ligand activated VDR forms a complex with VDR capable of modulating transcription at specific regions of called vitamin D response elements (VDREs). (a) Transcriptional activation is mediated by a number of coactivators and mediators, which function to initiate (b) or repress (c) the recruitment of transcriptional machinery. (d) Non-genomic, rapid actions of 1α,25(OH)2D3 are hypothesized to involve binding a cytosolic and membraneVDR.). Deeb, K.K., D.L. Trump, and C.S. Johnson, *Vitamin D signalling pathways in cancer: potential for anticancer therapeutics.* Nat Rev Cancer, 2007. **7**(9): p. 684-700.


A
OKF6-D1 + vit D
OKF6
+ vit D
OKF6-D1
OKF6
-1.7
0
1.7
Standardized Intensity

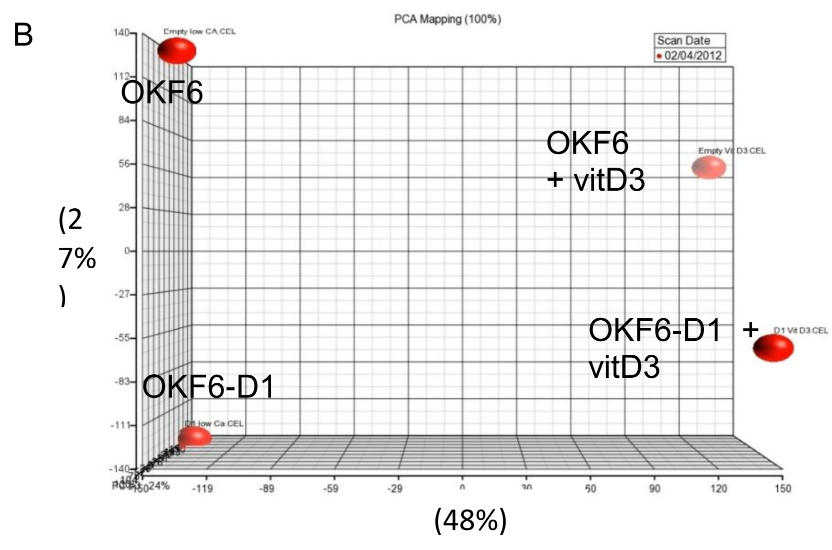

B
PCA Mapping (100%)
Scan Date
02/04/2012
Empty low CA.CEL
OKF6
OKF6
+ vitD3
Empty Vit D3.CEL
OKF6-D1 +
vitD3
D1 Vit D3.CEL
OKF6-D1
D1 low Ca.CEL
(2
7%
)
140
112
84
56
28
0
-27
-55
-83
-111
-140
-119
-89
-59
-29
50
90
120
150
(48%)

Figure 4. **Microarray analysis of vitamin D induced gene expression.** (a) Hierarchical clustering of OKF6 and OKF6-D1 keratinoctyes treated with vitamin D. Each row represents each sample tested and each column represents a single gene probe. Relative gene expression is color represented: red indicates higher gene expression, blue is lower, and grey is no change. The heat-map displays 770 differentially expressed with greater than or equal to a 3 fold change in gene expression (inclusion criteria). (b) Principal Component Analysis (PCA) of vitamin D induced gene expression in keratinocytes overexpressing cyclin D1 vs normal keratinocytes. PCA is a 3D scatter plot used to visual display the differences in global expression profiles with and following vitamin D treatment. Analysis of 4 samples resulted in 4 relatively distinct components of gene expression.

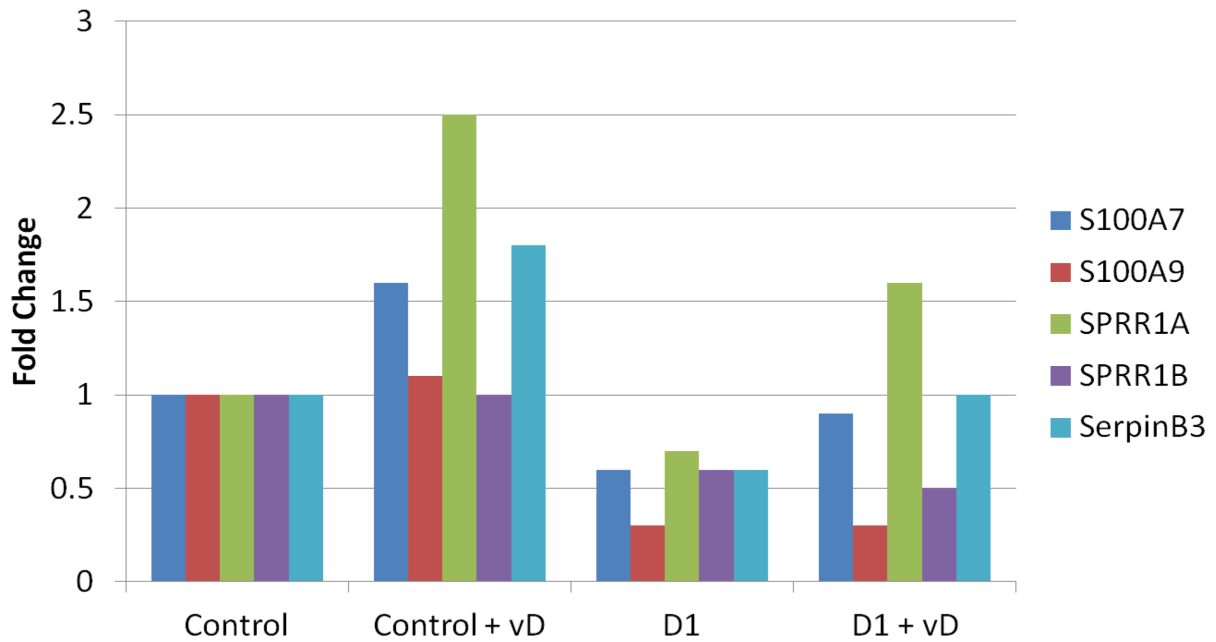


Figure 5. **Cyclin D1 partially inhibits vitamin D's induction of cornified envelope proteins.** RT-PCR analysis of OKF6and OKF6-D1 over-expressing keratinocytes treated with vitamin D. Gene candidates in the pathway of differentiation were found to be highly differentially expressed by microarray analysis. These candidate genes were validated with RT-PCR, and the expression levels of OKF6 cells were used as a baseline for comparison.

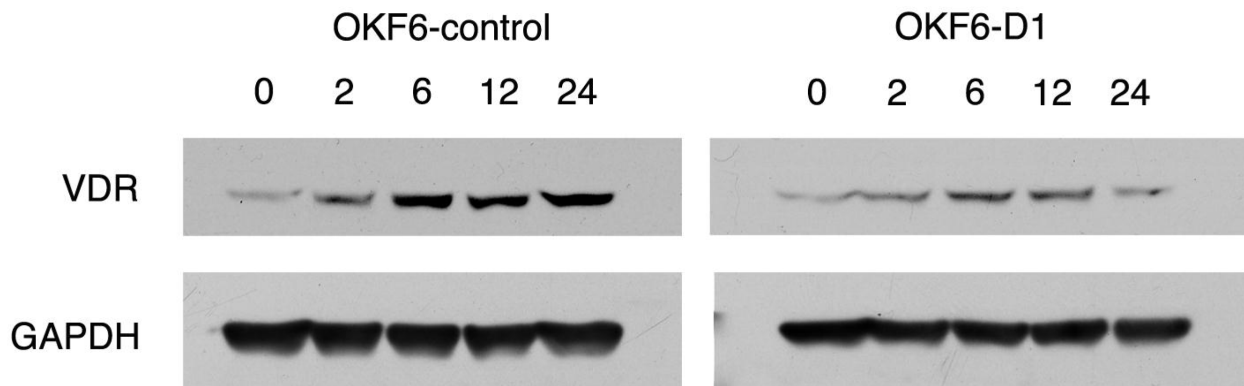


Figure 6. **Vitamin D Induces VDR.** Oral keratinocytes overexpressing cyclin D1 (D1) and OKF6keratinocytes show a vitamin D induced expression of VDR that gradual rises from 0 to 2 hours and peaks at 6 hours. VDR protein levels remain elevated at after 24 hours of treatment in OKF6cells, but declines in cyclin D1 over-expressing keratinocytes.

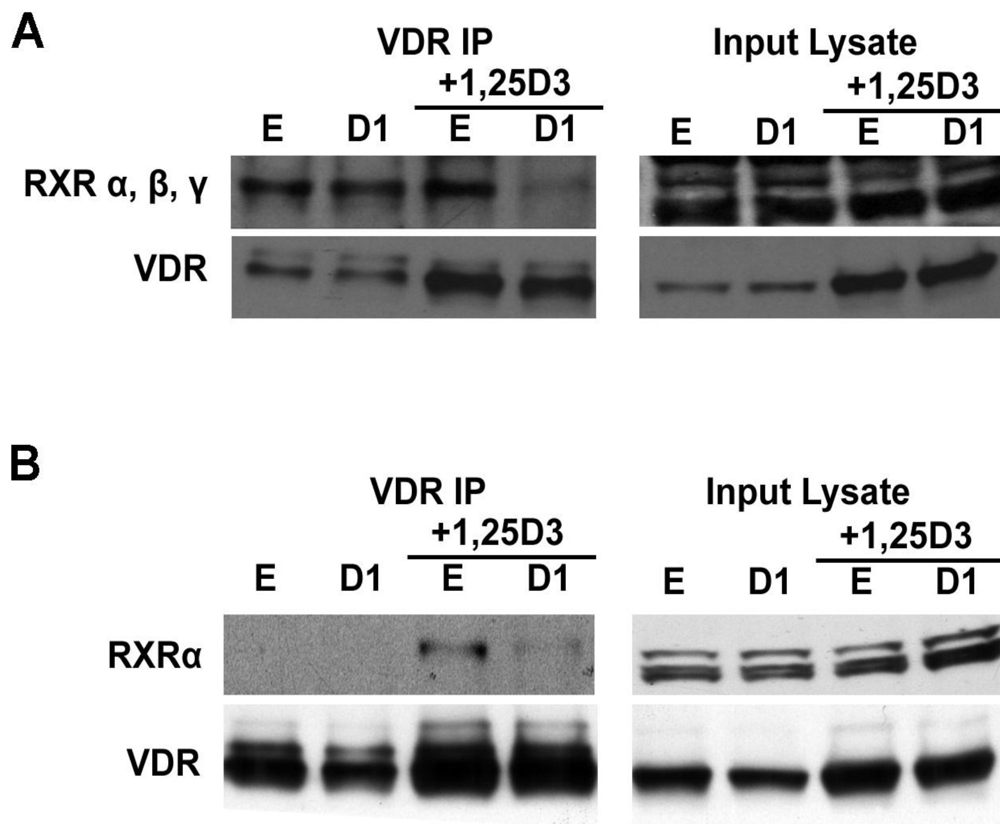
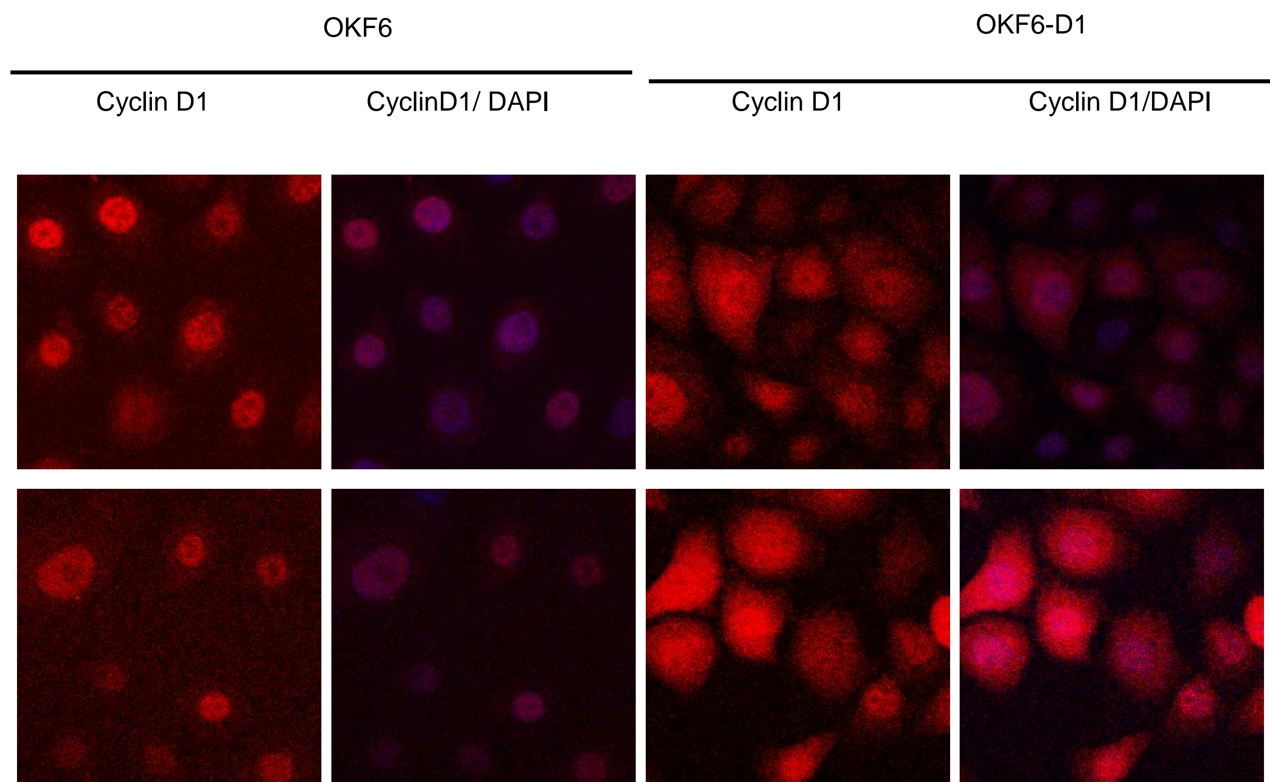
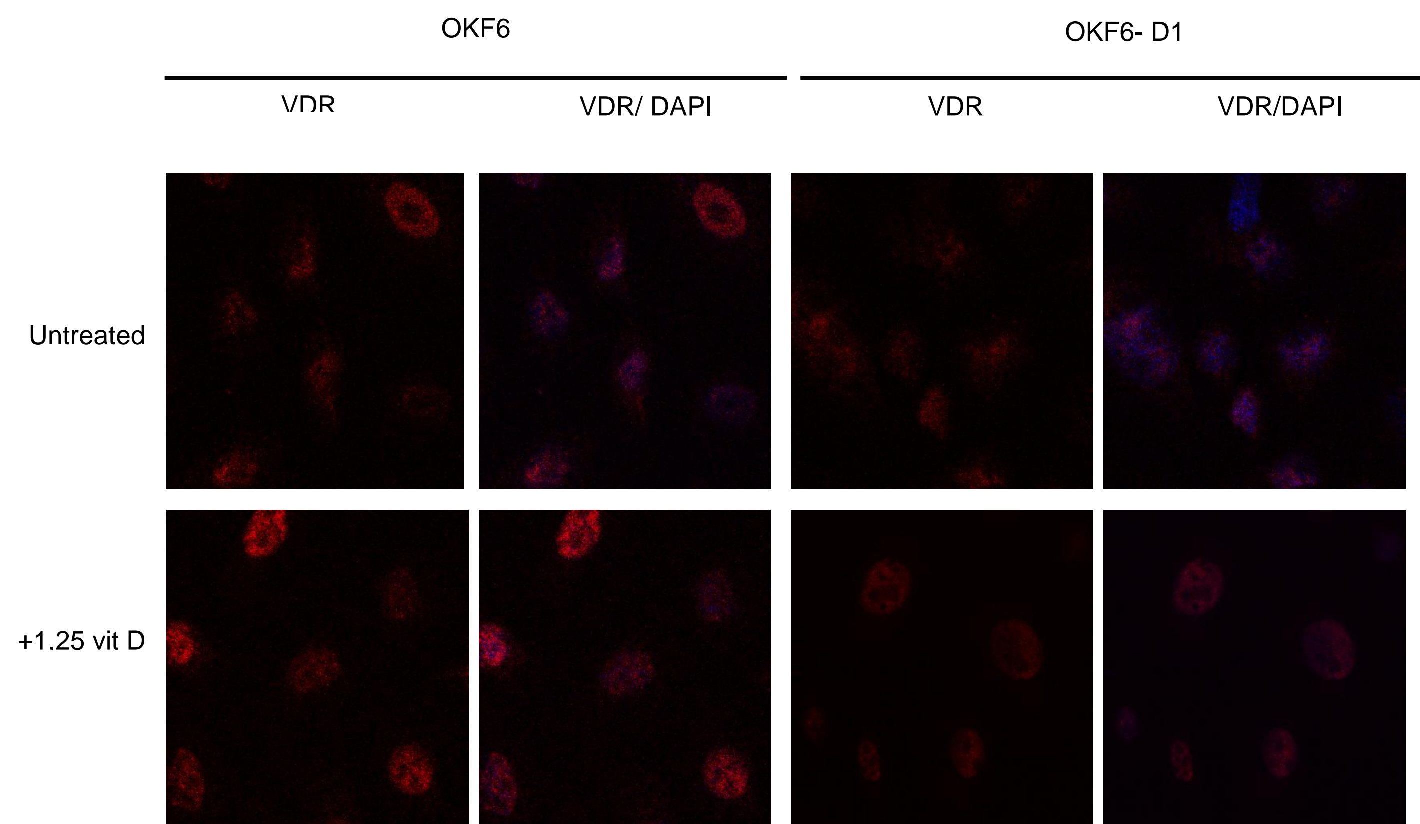


Figure 7. **Cyclin D1 blocks VDR–RXR binding.** OKF6-D1and OKF6keratinocytes were treated with $1,25(OH)_2D_3$ for 6h. Lysates were immunoprecipitated with an anti-VDR antibody (clone 9A7) and immunoblotted with an antibody against all 3 isoforms (fig. 7A), RXRα alone (fig. 7B) or VDR (clone D-6).

A




B
OKF6
OKF6- D1
VDR
VDR/ DAPI
VDR
VDR/DAPI
Untreated
+1.25 vit D

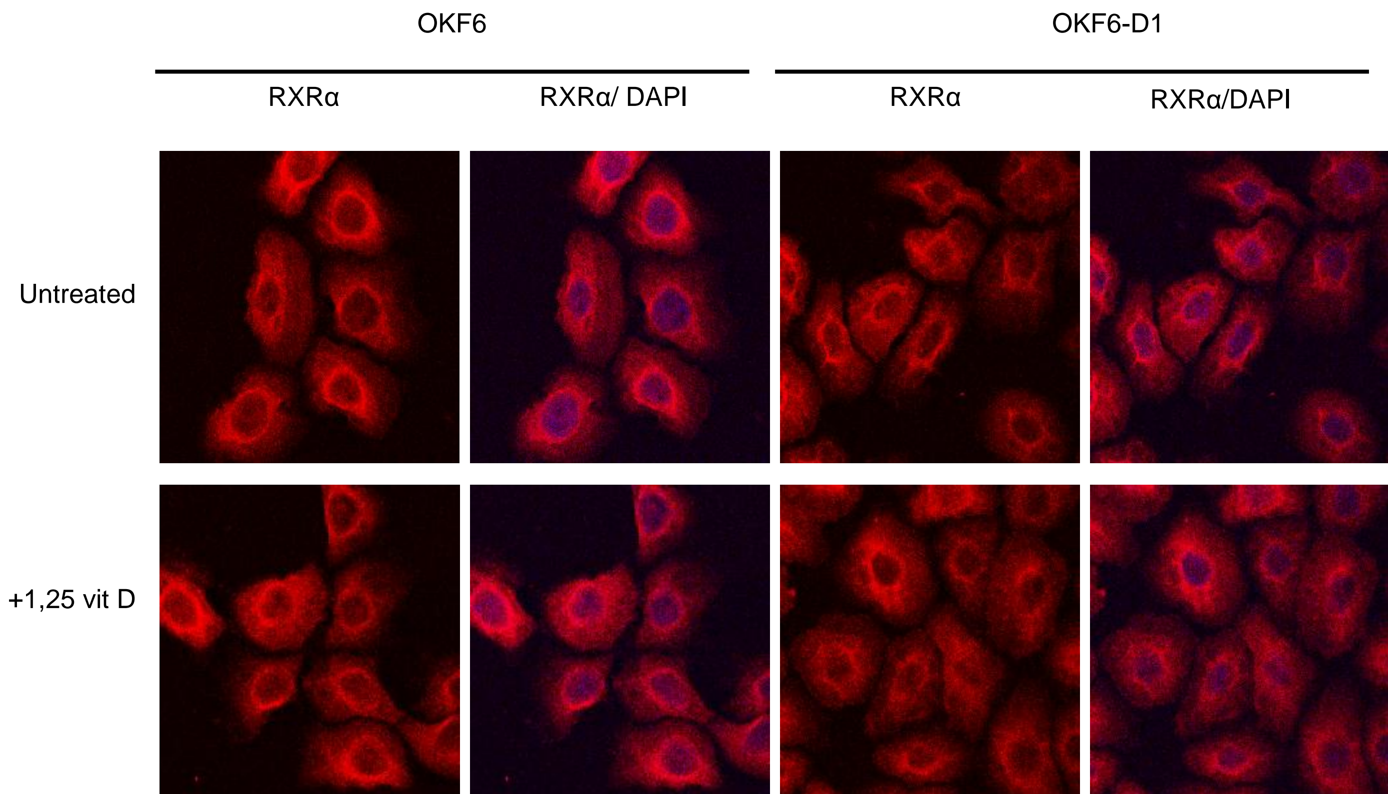


Figure 8. **Cyclin D1 over-expression does not alter vitamin D induced changes in localization.** Cells were treated with the 1,25$(OH)_2D_3$, 10^{-7}M for 6h. VDR [A], RXRα [B] and cyclin D1 [C] were detected by immunofluorescence. Nuclei were counterstained with DAPI and visualized under a confocal microscope. Overlay images were generated to visualize and differentiate nuclear and cytoplasmic staining.

References:

1. Parkin, D.M., et al., *Global cancer statistics, 2002.* CA Cancer J Clin, 2005. **55**(2): p. 74-108.
2. United States. Dept. of Health and Human Services. and National Institute of Dental and Craniofacial Research (U.S.), *Oral health in America : a report of the Surgeon General*. NIH publication ; no. 00-4713. 2000, Rockville, Md.: U.S. Public Health Service, Dept. of Health and Human Services. xxiv, 308 p.
3. Bikle, D.D., et al., *1,25-Dihydroxyvitamin D3 production by human keratinocytes. Kinetics and regulation.* J Clin Invest, 1986. **78**(2): p. 557-66.
4. Bikle, D.D., et al., *Neonatal human foreskin keratinocytes produce 1,25-dihydroxyvitamin D3.* Biochemistry, 1986. **25**(7): p. 1545-8.
5. Matsumoto, K., et al., *Involvement of endogenously produced 1,25-dihydroxyvitamin D-3 in the growth and differentiation of human keratinocytes.* Biochim Biophys Acta, 1991. **1092**(3): p. 311-8.
6. Lehmann, B., et al., *UVB-induced conversion of 7-dehydrocholesterol to 1alpha,25-dihydroxyvitamin D3 in an in vitro human skin equivalent model.* J Invest Dermatol, 2001. **117**(5): p. 1179-85.
7. Jones, G., S.A. Strugnell, and H.F. DeLuca, *Current understanding of the molecular actions of vitamin D.* Physiol Rev, 1998. **78**(4): p. 1193-231.
8. Horst, R.L., J.P. Goff, and T.A. Reinhardt, *Calcium and vitamin D metabolism during lactation.* J Mammary Gland Biol Neoplasia, 1997. **2**(3): p. 253-63.
9. Prosser, D.E. and G. Jones, *Enzymes involved in the activation and inactivation of vitamin D.* Trends Biochem Sci, 2004. **29**(12): p. 664-73.
10. Zierold, C., H.M. Darwish, and H.F. DeLuca, *Two vitamin D response elements function in the rat 1,25-dihydroxyvitamin D 24-hydroxylase promoter.* J Biol Chem, 1995. **270**(4): p. 1675-8.
11. Zella, L.A., et al., *Enhancers located within two introns of the vitamin D receptor gene mediate transcriptional autoregulation by 1,25-dihydroxyvitamin D3.* Mol Endocrinol, 2006. **20**(6): p. 1231-47.
12. Costa, E.M., M.A. Hirst, and D. Feldman, *Regulation of 1,25-dihydroxyvitamin D3 receptors by vitamin D analogs in cultured mammalian cells.* Endocrinology, 1985. **117**(5): p. 2203-10.
13. Mangelsdorf, D.J. and R.M. Evans, *The RXR heterodimers and orphan receptors.* Cell, 1995. **83**(6): p. 841-50.
14. Haussler, M.R., et al., *The nuclear vitamin D receptor: biological and molecular regulatory properties revealed.* J Bone Miner Res, 1998. **13**(3): p. 325-49.
15. McDonnell, D.P., et al., *Molecular cloning of complementary DNA encoding the avian receptor for vitamin D.* Science, 1987. **235**(4793): p. 1214-7.
16. Baker, A.R., et al., *Cloning and expression of full-length cDNA encoding human vitamin D receptor.* Proc Natl Acad Sci U S A, 1988. **85**(10): p. 3294-8.
17. Brumbaugh, P.F. and M.R. Haussler, *1 Alpha,25-dihydroxycholecalciferol receptors in intestine. I. Association of 1 alpha,25-dihydroxycholecalciferol with intestinal mucosa chromatin.* J Biol Chem, 1974. **249**(4): p. 1251-7.
18. Brumbaugh, P.F. and M.R. Haussler, *1 Alpha,25-dihydroxycholecalciferol receptors in intestine. II. Temperature-dependent transfer of the hormone to chromatin via a specific cytosol receptor.* J Biol Chem, 1974. **249**(4): p. 1258-62.

19. Rochel, N., et al., *The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand.* Mol Cell, 2000. **5**(1): p. 173-9.
20. Vanhooke, J.L., et al., *Molecular structure of the rat vitamin D receptor ligand binding domain complexed with 2-carbon-substituted vitamin D3 hormone analogues and a LXXLL-containing coactivator peptide.* Biochemistry, 2004. **43**(14): p. 4101-10.
21. Mangelsdorf, D.J., et al., *The nuclear receptor superfamily: the second decade.* Cell, 1995. **83**(6): p. 835-9.
22. Smith, C.L. and B.W. O'Malley, *Coregulator function: a key to understanding tissue specificity of selective receptor modulators.* Endocr Rev, 2004. **25**(1): p. 45-71.
23. Ozono, K., et al., *The vitamin D-responsive element in the human osteocalcin gene. Association with a nuclear proto-oncogene enhancer.* J Biol Chem, 1990. **265**(35): p. 21881-8.
24. Sutton, A.L. and P.N. MacDonald, *Vitamin D: more than a "bone-a-fide" hormone.* Mol Endocrinol, 2003. **17**(5): p. 777-91.
25. Deeb, K.K., D.L. Trump, and C.S. Johnson, *Vitamin D signalling pathways in cancer: potential for anticancer therapeutics.* Nat Rev Cancer, 2007. **7**(9): p. 684-700.
26. Krishnan, A.V., et al., *The role of vitamin D in cancer prevention and treatment.* Endocrinol Metab Clin North Am, 2010. **39**(2): p. 401-18, table of contents.
27. Trump, D.L., K.K. Deeb, and C.S. Johnson, *Vitamin D: considerations in the continued development as an agent for cancer prevention and therapy.* Cancer J, 2010. **16**(1): p. 1-9.
28. Beer, T.M., et al., *High-dose weekly oral calcitriol in patients with a rising PSA after prostatectomy or radiation for prostate carcinoma.* Cancer, 2003. **97**(5): p. 1217-24.
29. Gross, C., et al., *Treatment of early recurrent prostate cancer with 1,25-dihydroxyvitamin D3 (calcitriol).* J Urol, 1998. **159**(6): p. 2035-9; discussion 2039-40.
30. Trump, D.L., et al., *Phase II trial of high-dose, intermittent calcitriol (1,25 dihydroxyvitamin D3) and dexamethasone in androgen-independent prostate cancer.* Cancer, 2006. **106**(10): p. 2136-42.
31. Beer, T.M. and A. Myrthue, *Calcitriol in cancer treatment: from the lab to the clinic.* Mol Cancer Ther, 2004. **3**(3): p. 373-81.
32. Beer, T.M., et al., *Phase I study of weekly DN-101, a new formulation of calcitriol, in patients with cancer.* Cancer Chemother Pharmacol, 2007. **59**(5): p. 581-7.
33. Fakih, M.G., et al., *A phase I pharmacokinetic and pharmacodynamic study of intravenous calcitriol in combination with oral gefitinib in patients with advanced solid tumors.* Clin Cancer Res, 2007. **13**(4): p. 1216-23.
34. Muindi, J.R., et al., *Pharmacokinetics of high-dose oral calcitriol: results from a phase 1 trial of calcitriol and paclitaxel.* Clin Pharmacol Ther, 2002. **72**(6): p. 648-59.
35. Osborn, J.L., et al., *Phase II trial of oral 1,25-dihydroxyvitamin D (calcitriol) in hormone refractory prostate cancer.* Urol Oncol, 1995. **1**(5): p. 195-8.
36. Wilding, G., et al., *Results from a pilot Phase I trial of gefitinib combined with docetaxel and estramustine in patients with hormone-refractory prostate cancer.* Cancer, 2006. **106**(9): p. 1917-24.
37. Beer, T.M., et al., *Double-blinded randomized study of high-dose calcitriol plus docetaxel compared with placebo plus docetaxel in androgen-independent prostate cancer: a report from the ASCENT Investigators.* J Clin Oncol, 2007. **25**(6): p. 669-74.
38. Scher, H.I., et al., *Randomized, open-label phase III trial of docetaxel plus high-dose calcitriol versus docetaxel plus prednisone for patients with castration-resistant prostate cancer.* J Clin Oncol, 2011. **29**(16): p. 2191-8.

39. Rassnick, K.M., et al., *In vitro and in vivo evaluation of combined calcitriol and cisplatin in dogs with spontaneously occurring tumors.* Cancer Chemother Pharmacol, 2008. **62**(5): p. 881-91.
40. Abe-Hashimoto, J., et al., *Antitumor effect of 22-oxa-calcitriol, a noncalcemic analogue of calcitriol, in athymic mice implanted with human breast carcinoma and its synergism with tamoxifen.* Cancer Res, 1993. **53**(11): p. 2534-7.
41. Sternberg, C.N., et al., *Chemotherapy for bladder cancer: treatment guidelines for neoadjuvant chemotherapy, bladder preservation, adjuvant chemotherapy, and metastatic cancer.* Urology, 2007. **69**(1 Suppl): p. 62-79.
42. Rashid, S.F., et al., *Synergistic growth inhibition of prostate cancer cells by 1 alpha,25 Dihydroxyvitamin D(3) and its 19-nor-hexafluoride analogs in combination with either sodium butyrate or trichostatin A.* Oncogene, 2001. **20**(15): p. 1860-72.
43. Swami, S., et al., *Genistein potentiates the growth inhibitory effects of 1,25-dihydroxyvitamin D3 in DU145 human prostate cancer cells: role of the direct inhibition of CYP24 enzyme activity.* Mol Cell Endocrinol, 2005. **241**(1-2): p. 49-61.
44. Woloszynska-Read, A., C.S. Johnson, and D.L. Trump, *Vitamin D and cancer: clinical aspects.* Best Pract Res Clin Endocrinol Metab, 2011. **25**(4): p. 605-15.
45. Hellstrom, E., et al., *Treatment of myelodysplastic syndromes with retinoic acid and 1 alpha-hydroxy-vitamin D3 in combination with low-dose ara-C is not superior to ara-C alone. Results from a randomized study. The Scandinavian Myelodysplasia Group (SMG).* Eur J Haematol, 1990. **45**(5): p. 255-61.
46. Petrini, M., et al., *Low-dose ARA-C and 1(OH) D3 administration in acute non lymphoid leukemia: pilot study.* Haematologica, 1991. **76**(3): p. 200-3.
47. Ferrero, D., et al., *Combined differentiating therapy for myelodysplastic syndromes: a phase II study.* Leuk Res, 1996. **20**(10): p. 867-76.
48. Slapak, C.A., et al., *Treatment of acute myeloid leukemia in the elderly with low-dose cytarabine, hydroxyurea, and calcitriol.* Am J Hematol, 1992. **41**(3): p. 178-83.
49. Masuda, S. and G. Jones, *Vitamin D analogs--drug design based on proteins involved in vitamin D signal transduction.* Curr Drug Targets Immune Endocr Metabol Disord, 2003. **3**(1): p. 43-66.
50. Hansen, C.M., et al., *Seocalcitol (EB 1089): a vitamin D analogue of anti-cancer potential. Background, design, synthesis, pre-clinical and clinical evaluation.* Curr Pharm Des, 2000. **6**(7): p. 803-28.
51. Blot, W.J., et al., *Smoking and drinking in relation to oral and pharyngeal cancer.* Cancer Res, 1988. **48**(11): p. 3282-7.
52. Greenberg, R.S., et al., *The relation of socioeconomic status to oral and pharyngeal cancer.* Epidemiology, 1991. **2**(3): p. 194-200.
53. Brown, L.M., et al., *Family cancer history and susceptibility to oral carcinoma in Puerto Rico.* Cancer, 2001. **92**(8): p. 2102-8.
54. Giovannucci, E., et al., *Prospective study of predictors of vitamin D status and cancer incidence and mortality in men.* J Natl Cancer Inst, 2006. **98**(7): p. 451-9.
55. Lipworth, L., et al., *Dietary vitamin D and cancers of the oral cavity and esophagus.* Ann Oncol, 2009. **20**(9): p. 1576-81.
56. Bishop, J.M., *Cellular oncogenes and retroviruses.* Annu Rev Biochem, 1983. **52**: p. 301-54.
57. Duesberg, P.H., *Retroviral transforming genes in normal cells?* Nature, 1983. **304**(5923): p. 219-26.
58. Cooper, G.M., *Cellular transforming genes.* Science, 1982. **217**(4562): p. 801-6.
59. Weinberg, R.A., *The action of oncogenes in the cytoplasm and nucleus.* Science, 1985. **230**(4727): p. 770-6.

60. Leder, P., et al., *Translocations among antibody genes in human cancer.* Science, 1983. **222**(4625): p. 765-71.
61. Wong, A.J., et al., *Gene amplification of c-myc and N-myc in small cell carcinoma of the lung.* Science, 1986. **233**(4762): p. 461-4.
62. Tabin, C.J., et al., *Mechanism of activation of a human oncogene.* Nature, 1982. **300**(5888): p. 143-9.
63. Taparowsky, E., et al., *Activation of the T24 bladder carcinoma transforming gene is linked to a single amino acid change.* Nature, 1982. **300**(5894): p. 762-5.
64. Schwab, M., et al., *Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour.* Nature, 1983. **305**(5931): p. 245-8.
65. Kohl, N.E., et al., *Transposition and amplification of oncogene-related sequences in human neuroblastomas.* Cell, 1983. **35**(2 Pt 1): p. 359-67.
66. Seeger, R.C., et al., *Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas.* N Engl J Med, 1985. **313**(18): p. 1111-6.
67. Little, C.D., et al., *Amplification and expression of the c-myc oncogene in human lung cancer cell lines.* Nature, 1983. **306**(5939): p. 194-6.
68. Nau, M.M., et al., *L-myc, a new myc-related gene amplified and expressed in human small cell lung cancer.* Nature, 1985. **318**(6041): p. 69-73.
69. Gollin, S.M., *Chromosomal alterations in squamous cell carcinomas of the head and neck: window to the biology of disease.* Head Neck, 2001. **23**(3): p. 238-53.
70. Koontongkaew, S., et al., *Alterations of p53, pRb, cyclin D(1) and cdk4 in human oral and pharyngeal squamous cell carcinomas.* Oral Oncol, 2000. **36**(4): p. 334-9.
71. Akervall, J.A., et al., *Amplification of cyclin D1 in squamous cell carcinoma of the head and neck and the prognostic value of chromosomal abnormalities and cyclin D1 overexpression.* Cancer, 1997. **79**(2): p. 380-9.
72. Bova, R.J., et al., *Cyclin D1 and p16INK4A expression predict reduced survival in carcinoma of the anterior tongue.* Clin Cancer Res, 1999. **5**(10): p. 2810-9.
73. Izzo, J.G., et al., *Dysregulated cyclin D1 expression early in head and neck tumorigenesis: in vivo evidence for an association with subsequent gene amplification.* Oncogene, 1998. **17**(18): p. 2313-22.
74. Woods, M., R. Pant, and S.M. Mallya, *Cyclin D1 and cyclin D-dependent kinases enhance oral keratinocyte proliferation but do not block keratinocyte differentiation.* Int J Oncol, 2010. **37**(6): p. 1471-5.
75. Wilkey, J.F., et al., *Cyclin D1 overexpression increases susceptibility to 4-nitroquinoline-1-oxide-induced dysplasia and neoplasia in murine squamous oral epithelium.* Mol Carcinog, 2009. **48**(9): p. 853-61.
76. Coqueret, O., *Linking cyclins to transcriptional control.* Gene, 2002. **299**(1-2): p. 35-55.
77. Bernards, R., *CDK-independent activities of D type cyclins.* Biochim Biophys Acta, 1999. **1424**(2-3): p. M17-22.
78. Dickson, M.A., et al., *Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics.* Mol Cell Biol, 2000. **20**(4): p. 1436-47.
79. Qiagen, *RNeasy Micro Handbook*, Qiagen, Editor 2007, Qiagen. p. 55-59.
80. Zwijsen, R.M., et al., *CDK-independent activation of estrogen receptor by cyclin D1.* Cell, 1997. **88**(3): p. 405-15.

81. Comstock, C.E., et al., *Cyclin D1 is a selective modifier of androgen-dependent signaling and androgen receptor function.* J Biol Chem, 2011. **286**(10): p. 8117-27.