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Molecular Actions of the Vitamin D Receptor in Mammary and Skin Carcinogenesis

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Molecular actions of the vitamin D receptor in mammary and skin carcinogenesis.

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Abstract:

Vitamin D has been linked to breast cancer risk in animal and human studies, suggesting that the active metabolite 1,25-Dihydroxyvitamin D (1,25D) might protect breast cells from transformation. In human mammary epithelial (HME-hTERT) cells, which express vitamin D receptor (VDR), 1,25D exerts anti proliferative and pro-differentiating effects, but the molecular mechanisms that mediate these actions are unknown. In previous studies we used genomic profiling to classify 1,25D regulated networks in HME-hTERT cells that may contribute to the anti-cancer effects of vitamin D. Through this approach we detected a 60-fold increase in the cytokine CD14 in HME-hTERT cells treated with vitamin D. CD14 is a component of the innate immune system which also functions in apoptotic cell clearance and mammary gland remodeling. The studies described in the first part of this thesis were designed to confirm and extend this preliminary data. HME-hTERT cells, which were used to generate the preliminary data, and HME-PR cells, a transformed derivative, and HC11 cells, mouse mammary epithelial cells, were cultured and treated with vitamin D metabolites (25-hydroxyvitamin D and 1,25-dihydroxyvitamin D). Doses ranged from 0-100nM, and duration of treatment was 24h. qPCR was used to analyze the expression of CD14 and related immune response genes. These studies determined the extent to which 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D alter the synthesis or secretion of cytokines in mammary cells (both human and mouse) and how transformation alters the basal and vitamin D-induced expression of these cytokines. Our data suggest that vitamin D regulation of cytokines is altered during the process of carcinogenesis and that the specific targets of VDR that regulate immune responses differ in human and mouse cells.

In addition to effects on immune responses, the anti-cancer effects of vitamin D have been linked to modulation of the extraceullular matrix. In previous studies, the Welsh lab identified Has2 (hyaluronic acid synthase 2) as a vitamin D regulated gene in invasive murine mammary cancer cells. Has2 is the rate-limiting enzyme in the synthesis of hyaluronic acid (HA), which accumulates in the extracellular matrix. Has2 over-expression and HA synthesis have been linked to cancer cell invasion and metastasis in vitro and abnormal skin phenotypes in vivo. The abnormal skin phenotype associated with Has2 over-expression is highly similar to skin changes reported in VDR knockout (VDRKO) mice, suggesting that Has2 regulation by VDR may have functional consequences in vivo. In the second part of this thesis project, we further investigated the link between VDR and Has2 expression/function in skin and mammary tissue of mice. Tissues from wildtype and VDRKO mice of different ages were sectioned and processed for Has2 (immunofluorescence), HA (HABP binding assay) and proteoglycans (alcian blue staining). Additional in vitro studies were conducted to establish western blotting and PCR assays for study of this pathway. Our data suggest that vitamin D and the VDR physiologically regulate Has2 and HA production in vivo, especially in the epidermis.

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I. INTRODUCTION

Vitamin D is essential for growth and development in most biological systems. There are two naturally occurring forms of vitamin D—vitamin D_2 and vitamin D_3. Vitamin D_2 is synthesized in plants while D_3 is synthesized in animals. The vitamin D present in humans can be generated by UVB radiation or can be obtained from the diet and supplements. UVB radiation converts 7-dehydrocholesterol in the skin to pre-vitamin D_3 which, through a heat dependent process, isomerizes into vitamin D_3. Once generated, a vitamin D binding protein (DBP), which is present in serum, binds vitamin D_3 and transports it to the liver. In the liver, vitamin D_3 is converted to 25-hydroxyvitamin D by the enzyme 25-hydroxylase. 25-hydroxyvitamin D_3 is then circulated to the kidneys to be converted into the active form 1,25-dihydroxyvitamin D (1,25 D) by the 1-alpha hydroxylase enzyme. The biological actions of vitamin D are mediated by 1,25D which is the ligand for the vitamin D receptor (VDR). The VDR belongs to the nuclear receptor family which are trans-acting transcriptional regulatory factors. The VDR heterodimerizes with the retinoid X-receptor (RXR) and this complex binds to vitamin D response elements on DNA which modulates the expression of target genes (Kato 2000). Once the VDR is liganded and bound to RXR the complex activates or represses specific target genes in each tissue.

Vitamin D's best characterized function is the regulation of calcium and phosphorus absorption which is essential for maintenance of muscle and bone health. Yet vitamin D also functions as an immunomodulator which contributes to the prevention of autoimmune diseases and the control of invading pathogens. In addition, vitamin D regulates cell growth and differentiation in many epithelial tissues including the mammary gland and skin Many epidemiological

studies have demonstrated that high vitamin D status correlates with reduced risk for chronic diseases including cancer.

The immune regulatory effects of vitamin D have been demonstrated in animal models of autoimmune disease (Deluca and Cantorna 2001). In these studies, both vitamin D deficiency and the absence of the VDR enhanced disease development and severity. Other reports have demonstrated anti-inflammatory effects of vitamin D through effects on macrophages and other immune cells. However, very few studies have examine whether vitamin D alters immune genes in epithelial cells. Interestingly, microarray analysis in the lab of my mentor Dr JoEllen Welsh, indicated that immune regulatory genes were up regulated in human mammary epithelial (HME-hTERT) cells treated with 100nM of 1,25 D for 24 hours. Some of the immune regulatory genes that were identified in the Welsh studies include CD14, cathelicidin (CAMP), defensin β1 (DEFB1), and select interleukins (IL). In the first part of my thesis work, I conducted experiments to validate and extend these data on regulation of immune genes in mammary cells treated with vitamin D.

The anti-cancer effects of vitamin D have also been well studied at the cell and molecular level. *In vitro* and *in vivo* studies have consistently demonstrated that vitamin D exerts anti-proliferative and pro-apoptotic effects on cancer cells (Daniellson et al 1984). Cancer cell models have been developed in the Welsh lab to study the mechanisms by which vitamin D inhibits proliferation. One model employs transformed mouse mammary cells derived from wild-type (WT) and VDR knockout (VDRKO) mice. In this model system, microarray analysis found that the expression of the hyaluronan synthase 2 (Has2) gene was down regulated by 1,25 D (100nM, 24h). Has2 is an enzyme that synthesizes hyaluronic acid (HA), an extracellular glycan

which activates cell proliferation and has been linked to cancer progression in many studies (Matthews et al 2010). In the second part of my project I focused on the regulation of Has2 by vitamin D in cell and animal models of cancer.

The overall goal of my project was to further elucidate the role of vitamin D in normal and cancerous cells and tissues. In the first series of studies, I measured the effect of vitamin D metabolites on the expression of select immunological genes in normal human (HME-hTERT cells) and mouse (HC-11 cells) cell lines and in a cancerous variant of the HME-hTERT cells (HME-PR cells). The studies were designed to test the hypothesis that the induction of the immunological genes CAMP, CD14, DEFB1, IL1A, and IL1B by vitamin D would be similar in the normal cell lines derived from human and mouse, but would be reduced in the cancerous HME-PR cells. In the second series of studies, I investigated the link between the VDR and Has2 *in vivo* using mammary and skin tissues and cells from WT and VDRKO mice. These studies were designed to test the hypothesis that the expression of Has 2 would be deregulated in the tissue of VDRKO mice compared to WT mice.

II. EFFECTS OF VITAMIN D ON CYTOKINE SYNTHESIS IN NORMAL AND TRANSFORMED MAMMARY EPITHELIAL CELLS.

a. Rationale for Study

Genomic profiling of mammary epithelial cells demonstrated a cohort of immune regulatory genes that were altered by 1,25D. Several of these genes [CD14, CAMP, DEFB1 and ILs (IL1A, IL1B, IL10, IL-6)] are important mediators of the innate immune response and were chosen for follow-up studies. CD14 plays a major role in innate immunity through interaction with the toll like receptor 4 (TLR4). CD14 is a pattern recognition receptor that binds lipopolysaccharide (LPS). There are two forms of CD14; soluble CD14 and membrane CD14. Both forms of CD14 bind LPS and transport it to TLR4 which activates the TLR4 signaling pathway activating a pro-inflammatory cellular response. TLR4 is a protein that detects liposaccharides in the gram-negative bacteria; thus it helps activate the innate immune system.

CAMP is an antimicrobial polypeptide that can be found in lysosomes, macrophages, and leukocytes. The CAMP polypeptide is activated by bacteria, viruses, fungi, or 1,25-D. CAMP primarily functions as an innate immune defense against bacterial infection. DEFB1 is an antimicrobial protein that is a part of the defensin family of cytotoxic peptides. DEFB1 and other defensins are cationic proteins. Defensins function by impeding gene expression and preventing viruses from entering their target cells.

Interleukins (IL) are produced primarily by macrophages in the body as proproteins. Proproteins are proteins that are cleaved to form an active protein; the ability to do this can be turned on and off. IL1A and IL1B play an important role in the pro-inflammatory immune response by

mediating cell differentiation, proliferation, and apoptosis. These cytokines are able to slow the rate of proliferation of cells and to repress the half-life of mRNA. IL6 plays a role in both the pro-inflammatory and anti-inflammatory response; it is found in T cells, B cells, monocytes, and PMNs. It is known to induce the acute-phase protein response when acting in the pro-inflammatory immune system. When acting as an anti-inflammatory gene it is known to inhibit TNF and IL1 production by macrophages (Opal et al, 2004). IL10 is a major player with regards to the anti-inflammatory immune response. It comes from monocytes and macrophages, and T and B cells. IL10 inhibits monocyte/macrophage and neutrophil cytokine productions as wells as inhibiting lymphocyte response (Opal et al, 2004).

b. Materials and Methods

The HME-hTERT cells and the HME-PR cells were split and cultured in 171 Media (Gibco) containing growth supplement (EGF, insulin, hydro-cortisone and pituitary extract). The HME-PR cells were plated in well plates and treated with 1,25 D or 25D and vehicle (ethanol) for 24 hours. After treatment, RNA was harvested from the HME-PR cells following an established protocol using the kit reagents: Buffer RLT, Buffer RW1, and Buffer RPE.

The HC11 cells were split and cultured in RPMI Media which contains 250uL 20ng/uL Mouse EGF, 500uL of 50 mg/mL Gentamicin, 5mL 200mM Glutamine (Glutamax), and 50mL of Fetal Bovine Serum (FBS) per 500mL of media. 50uL of insulin was added for every 10mL of media. The HC11 cells were plated in well plates and treated with a concentration ranging from 0-100nM 1,25D or 25D and vehicle (ethanol) for 24 hours. After treatment, RNA was harvested using the same established protocol used when harvesting HME-PR cell RNA.

After RNA was isolated, it was purified using the Qiagen RNeasy protocol. The protocol uses a silica-based membrane and high-salt buffers to isolate RNA longer than 200 bases. This protocol specifically selects for the messenger RNA (mRNA). The mRNA concentration and integrity was then measured using the nanodrop (NANODROP ND-1000 Spectrophotometer).

The isolated RNA was then used to prepare cDNA (complimentary DNA) through reverse transcription (RT) for polymerase chain reaction (PCR) analysis. Reverse transcription occurs when the purified RNA strand's complimentary base pairs are formed thus creating a DNA strand. There are three steps when preparing cDNA in the Mastercycler gradient machine (Eppendorf): the first brings the DNA to 25˚C for 10 minutes, the temperature then rises to 37˚C for one hour, and finally the temperature rises to 95˚C for five minutes. Once all steps are done, the samples are held at 4˚C. RT master mix includes RT Buffer, MgCl, dNTPs, Random Hexamers, RNAse Inhibitor, and Muiltiscribe RT.

The cDNA was then used for quantitative (q) PCR. qPCR is a method used for quantitating changes in gene expression of genes of interest. The genes of interest in this experiment were CD14, CAMP, DEFB1, IL1A, IL1B, IL6, IL10, and TLR4. 18S was also measured as a normalization control. The experimental primers targeting the genes of interest and SYBR green were used in this experiment. SYBR Green binds to DNA and fluoresces as the PCR product accumulates allowing for product to be detected and recorded. The 1,25 D and vehicle treated HME-PR cell cDNA samples were plated in duplicate in a 384 well plate. Once plating was done, RT PCR was performed using the 7900HT Fast Real-Time PCR system (Applied BioSystems). The thermocycler begins by bringing the plate with samples to 50˚C for two minutes. Then the temperature rises to 95˚C for 10 minutes. In the final step, the

temperature is kept at 95˚ for 15 seconds and then lowered to 60˚C for one minutes. These steps are repeated 40 times until finished. For each primer, gene expression was normalized to 18S expression. The data is presented as fold change in gene expression in treated samples (1,25D or 25D) relative to vehicle treated control samples.

c. Results

In this series of studies on vitamin D regulation of immune genes, we evaluated the effects of vitamin D on several genes that mediate innate immune responses. We first conducted comparative studies in normal and cancerous mammary cells. qPCR assays were used to assess gene expression in cells treated with 100nM 1,25D for 24 hours. Fold change for each gene was calculated and graphed.

Figure 1A displays the average fold change of the various genes tested—CAMP, CD14, DEFB1, IL1A, and IL1B—in non-cancerous HME-hTERT cells. Fold change was calculated by normalizing experimental data against the ethanol control. In the HME-HTERT cells, CAMP was induced 3-fold by 1,25D. CD14 was induced 232.2-fold by 1,25D. There were no significant differences in the expression of DEFB1, IL1A, and IL1B between vehicle and 1,25D treated cells.

Figure 1B displays the average fold change of the various genes tested—CAMP, CD14, DEFB1, IL1A, and IL1B—in HME-PR cells. In HME-PR cells, CAMP has the largest observed fold change at 13.33. CD14 has the next largest observed fold change at 9.97. No significant differences in DEFB1 or IL1A were detected between vehicle and 1,25D treated cells. IL1B was induced 6-fold in 1,25D treated cells compared to vehicle treated cells.

Figure 1C shows the average fold change for the same genes, except IL1A, in HME-PR cells treated with 100nM 25D. In response to 25D treatment CD14 was induced at 3-fold. CAMP and IL1B were induced 2-fold when treated with 25D. No significant difference was detected in DEFB1.

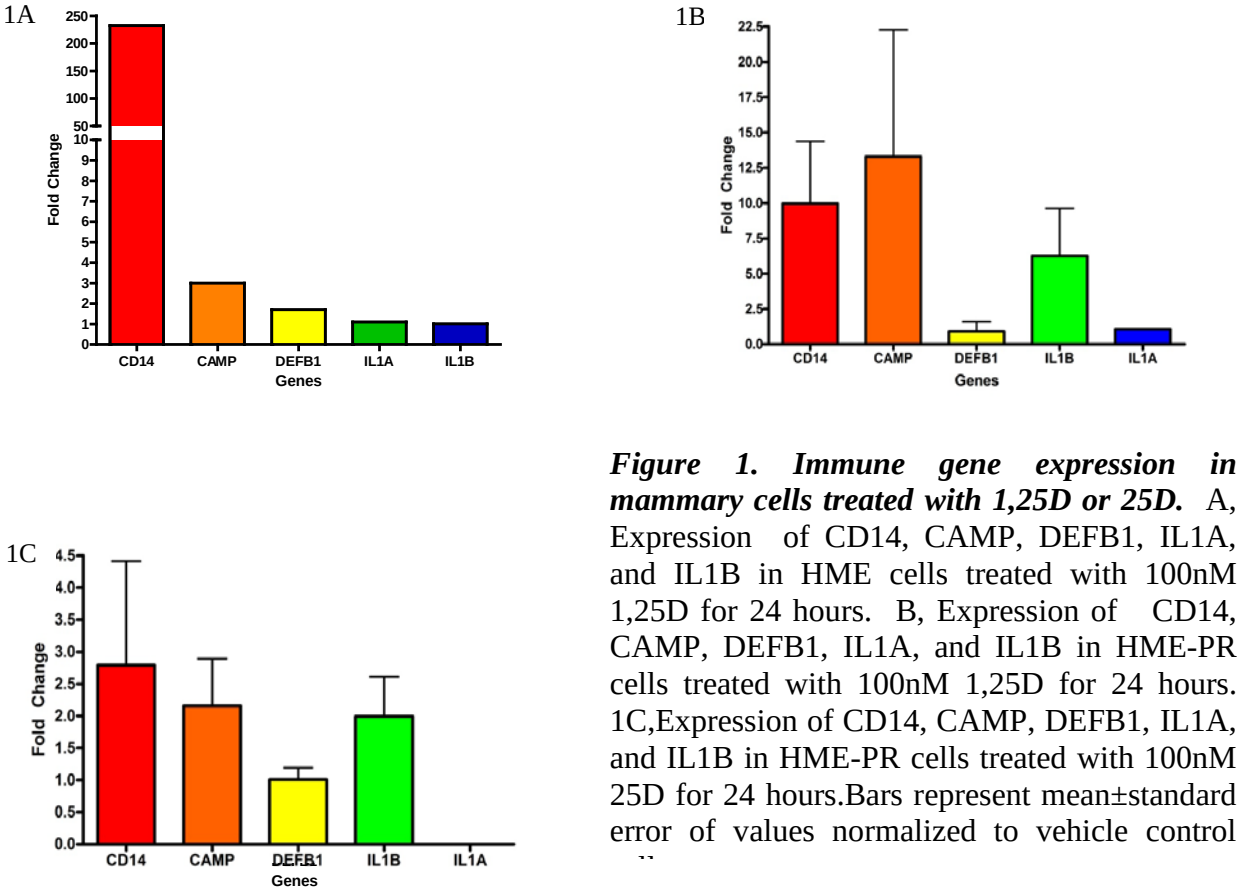

Figure 1. Immune gene expression in mammary cells treated with 1,25D or 25D. A, Expression of CD14, CAMP, DEFB1, IL1A, and IL1B in HME cells treated with 100nM 1,25D for 24 hours. B, Expression of CD14, CAMP, DEFB1, IL1A, and IL1B in HME-PR cells treated with 100nM 1,25D for 24 hours. 1C,Expression of CD14, CAMP, DEFB1, IL1A, and IL1B in HME-PR cells treated with 100nM 25D for 24 hours.Bars represent mean±standard error of values normalized to vehicle control

In order to determine whether mouse models would be useful in assessing vitamin D effects on immune responses in mammary gland, we next tested the effects of vitamin D compounds on immune gene expression in a mouse mammary epithelial cell line (HC11). As demonstrated in Figure 2, we tested the effects of both 1,25D (2A,C) and 25D (2B,D) in these studies. The results (Fig 2A) indicate trends for 1,25D (at concentrations of 25nM and above) to enhance the

expression of CD14, TLR4, and IL6 in the HC11 cells but these differences were less than 2-fold and were not statistically significant. Likewise, 25D at concentrations of 50nM and above appeared to enhance expression of CD14, TLR4 and IL-6 but these differences were not statistically significant. In contrast to these three genes which were marginally affected by vitamin D compounds, 1,25D dose dependently increased the expression of the anti-inflammatory cytokine IL10 in HC11 cells, with a 5-fold increase at 5nM, 12-fold at 25nM, 14-fold at 50nM and 17-fold at 100nM (Fig 2C). In cells treated with 25D, the induction of IL-10 was less than that seen with 1,25D (maximum induction of 2.6 fold was observed at 50nM).

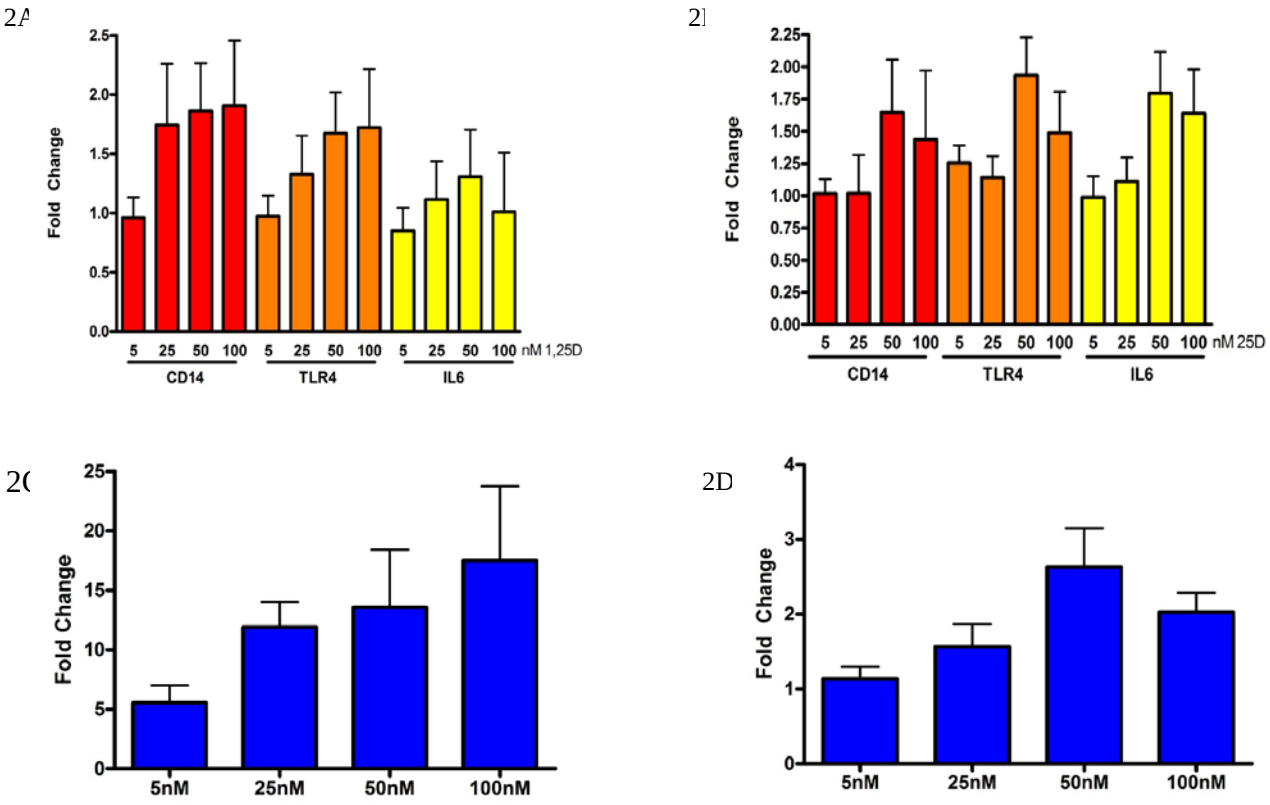

Figure 2. Regulation of cytokine gene expression by 1,25D and 25D in HC11 cells. A, Expression of CD14, TLR4, and IL6 in HC11 cells treated with 1,25D for 24 hours. B, Expression of CD14, TLR4, and IL6 in HC11 cells treated with 100nM 25 for 24 hours. C, Expression of IL10 in HC11 cells treated with 0-100nM 1,25D for 24 hours. D, Expression of IL10 in HC11 cells treated with 0-100nM 25D for 24 hours. Bars represent mean±standard error of values normalized to vehicle control cells.

d. Discussion

In these experiments, PCR analysis was conducted on various mammary epithelial cell lines (HME-hTERT cells, HME-PR cells and HC11 cells) to determine the effects of vitamin D on immune gene expression. HME-hTERT cells (human mammary epithelial cells) were derived from the mammary gland of a healthy female donor and represent a normal mammary cell line. HME-PR cells were generated from the HME-hTERT cell line through introduction of oncogenes leading to p53 disruption and Ras activation. These cells are invasive and tumorigenic and thus represent a cancerous cell line. HC11 cells were derived from mammary gland of BALB mice (Danielson et al, 1984) and represent the murine equivalent of the normal HME-hTERT cells. The immune genes studied in this PCR analysis were CD14, CAMP, DEFB1, IL1A, and IL1B (HME-hTERT and HME-PR cells), and CD14, TLR4, IL6, and IL10 (HC11 cells).

As seen in Figure 1, which compares immune gene expression in HME-hTERT cells versus HME-PR cells treated with vitamin D metabolites, the cancerous HME-PR cells are in general less sensitive to vitamin D mediated immune gene regulation. The data were calculated as fold change which refers to the difference in gene expression between ethanol (vehicle) treatment and vitamin D treatment within each cell line. The induction of CD14 gene expression in the HME-PR cells, both 1,25 D and 25D treated (10 fold change and 3 fold change respectively) was much less than that observed in HME-hTERT cells (200 fold change). DEFB1 gene expression was not increased in HME-PR cells treated with either 1,25D or 25D but was 2-fold increased in the HME-hTERT cells. IL1A was not altered in either cell line treated with 1,25D and therefore was not tested in 25D treated cells.

Exceptions to the general observation of reduced vitamin D mediated effects in HME-PR cells were seen with CAMP and IL1B gene expression. In HME-PR cells treated with 1,25D, CAMP was increased in 13 fold as compared to only 3 fold in HME-hTERT cells. Also, when HME-PR cells were treated with 25D, CAMP expression decreased 2 fold, as compared to change) was seen. For IL1B, gene expression was increased in the HME-PR cells (6 fold change: 1,25D treatment and 2 fold change: 25D treatment) but was unaffected in the HME –hTERT cells.

In the normal mouse mammary cell line HC11 treatment with varying doses of 1,25D for 24 hours only marginally increased CD14, TLR4 and IL-6, suggesting that these genes may not be under vitamin D control in murine cells. For most genes, only slight induction (less than 2-fold) was evident, begining at 25nM for 1,25D and 50nM for 25D. For both vitamin D metabolites these effects plateaued at doses greater than 50nM. Interestingly, IL10 was dose-dependently up-regulated by 1,25D (and to a lesser extent by 25D) in the HC11 cells with peak induction of 15-fold by 100nM 1,25D. Since IL-10 mediates anti-inflammatory effects, this observation may be relevant to the reported increased inflammation in the mammary gland of VDRKO mice (Welsh et al, 2010).

The genes examined in HC11 cells (CD14, TLR4, IL6, and IL10) were selected because they are known to be regulated by vitamin D in human cells. CD14 plays a major role in activating the TLR4 signaling pathway by binding to LPS. Once CD14 binds to LPS, the first line of cell defense—the TLR4 signaling pathway—is triggered, thus allowing for the innate immune system to be activated. IL6 is a known pro-inflammatory cytokine. On the other hand, IL10 is a known anti-inflammatory cytokine. If the TLR4 signaling pathway is activated by the binding of CD14, an up-regulation of IL6 would be found. However, if the TLR4 signaling pathway is not

activated, an anti-inflammatory response (in this case, by IL10) is induced. The limited up-regulation of CD14, TLR4, and IL6 by vitamin D in the HC11 cells, with concomitant up-regulation of IL-10, suggests that an anti-inflammatory response might be triggered in the presence of 1,25D. Since published work has indicated interaction between vitamin D and IL-10 in mouse models of colon inflammation, further studies would be of interest to examine this connection in mammary gland.

In summary, these are the first studies to compare the effects of vitamin D metabolites on immune response genes in normal and cancerous mammary cells and to examine whether these effects are conserved in human and mouse models. Although our results indicate some interesting trends that suggest altered immune gene regulation by 1,25D in cancer cells, and distinct target genes for VDR in human vs mouse cells, additional studies are needed to confirm these results. The experiments shown here need to be repeated due to high standard deviation. Furthermore, since all our studies were conducted after 24h treatment, it would be of interest to examine cells for longer periods of time This is especially important in the case of 25D because it requires enzymatic conversion into 1,25D for activity and this may not be complete within the 24h time frame used.

III. REGULATION OF HAS2 BY VDR IN MAMMARY GLAND AND EPIDERMIS

a. Rationale for Study

In a microarray screen of mouse breast cancer cells, the expression of the Hyaluronan synthase 2 (Has2) gene was down regulated by 1,25D (100nM, 24h). Has2 is an enzyme that, along with Has1 and Has3, synthesizes hyaluronic acid (HA). HA is known to play a major structural and functional role in extracellular matrices and body fluids by "binding to other matrix molecules like proteoglycans, thereby organizing the architecture and regulating the mechanical properties of tissues" (Rilla et al, 2010) such as cartilage. HA creates an optimal environment for cell growth and renewal. In addition, it can act as a signaling molecule in which it activates events in cells which "influence processes such as proliferation, migration, adhesion, apoptosis, cell shape, and multi-drug resistance" (Rilla et al, 2010). That is, HA can be linked to the proliferation of tumor cell lines and eventual cancer. HA is known to be degraded by hyaluronidases (Hyal) there are three isoforms —Hyal1, Hyal2, and Hyal 3. The amount of HA in individual tissues therefore depends on the relative expression and activity of three Has genes and three Hyal genes. Two tissues in which Has2 and/or HA have been consistently linked to proliferation and carcinogenesis are the skin and the mammary gland. Of relevance to this project, both skin and mammary gland of VDRKO mice display hyperproliferation and are highly susceptible to chemically induced carcinogenesis. Therefore our observation that vitamin D regulates Has2 in mouse breast cancer cells suggests that accumulation of HA may contribute to the abnormalities in mammary gland and skin that develop in VDRKO mice (Welsh, 2007). In support of this suggestion, genetic analyses have shown that the wrinkly skin phenotype in Shar Pei dogs, which resembles that of the VDRKO mice, results from duplication of the Has2 gene (Olsson et al, 2011).

b. Materials and Methods

In the first series of experiments we examined normal and cancerous tissues which were archived from previous studies on WT and VDRKO mice. For normal tissue, we analyzed mammary gland from mature adult female mice. For mammary tumors, mice were treated with the chemical carcinogen DMBA to induce tumor growth. Normal skin tissue was collected from adult male mice. All tissues were fixed in formalin and embedded in paraffin for sectioning. After sectioning was complete, three different staining protocols were used for detection. For the immunohistochemistry protocol to detect Has2, the tissues were first deparafinized and rehydrated. Next, endogenous peroxidase activity was quenched by treating with 3% hydrogen peroxide. Dako Protein Block was utilized to block for 10 minutes. Tissues were then incubated with the primary antibody for 1 hour at room temperature. Once incubation was complete, tissues were washed 3 times with PBS. After, tissues were incubated with Biotinylated Secondary from the VECTASTAIN kit for half an hour. Once again, tissues were washed 3 times with PBS. Tissues were then incubated for half an hour with ABC Reagent (from the VECTASTAIN kit). PBS was used to wash the tissues 3 times after completion. DAB chromogen was used on the tissues and allowed to develop to an appropriate stain. Tissues were then counterstained with hematoxylin. After counterstaining, tissues were dehydrated and mounted.

For the HA binding protein staining protocol, tissues were first deparafinized and rehydrated. Endogenous peroxidase activity was quenched by treatment with 3% hydrogen peroxide. When this was finished, tissues were rinsed with PBS. Next serum free blocking solution (Dako Protein block) was used. Slides were then incubated with Bovine HABP (bHABP) overnight at

4°C. The next day, slides were rinsed with PBS. Detection with the tertiary reagent of the VECTASTAIN Elite ABC kit was done. DAB substrate was then placed on tissue sections and allowed to develop to an appropriate stain. Sections were then rinsed, and counterstained with hematoxylin. After this was completed, sections were dehydrated and mounted.

The Alcian blue staining protocol was used to detect proteoglycans in mammary tissue sections only. Sections were first deparafinized and rehydrated. Once this was done, Alcian blue stain, pH 2.5, was used. Tissues were counterstained with hematoxylin. The tissue sections were then dehydrated and mounted.

In the second series of experiments, we utilized cell culture models to further explore the relationship between vitamin D and Has2. Cell lines included the WT145 and KO240 transformed mammary cells which were used for the original microarray screening and the HC11 cells as discussed in section II. The WT145 and KO240 cell lines were originally isolated from DMBA induced mammary tumors in wild type (WT) or VDRKO mice. For the KO^{hVDR} cells, the human VDR (HVDR) sequence was stably reintroduced into KO240 cells. The cell lines were treated with vitamin D as described below and were used to perform western blots and qPCR studies.

For western blots to detect the Has2 and VDR proteins, cells were treated with ethanol, 100nM vitamin D, 100nM all trans-retinoic acid (ATRA), or 100nM combo (1:1 mixture of vitamin D and ATRA). SDS-PAGE separating (7%) and stacking gels and running buffer were mixed and poured according to the standard protocols. The gels were loaded with the appropriate samples with the ladder in lane one and 1X Laemmli Buffer in lane 10. The gels were run at 80V until samples were well stacked. After this point, the voltage was increase to 130V and gels were

allowed to run until the dye line reached the end of the gel. To transfer the gels, two large sheets and four gel sized sheets of filter paper, and a gel sized sheet of PVDF membrane were cut and soaked in semi-dry blotting buffer. These were layered on the transfer unit in this order: two large filter papers, two small filter papers, western gel, transfer membrane, two small filter papers, and finally two large filter papers. Transfer was allowed to occur for about an hour at 500mAmps. After transfer was complete, the membrane was blocked for 1 hour in 5% skim milk at room temperature. Next, 500uL of the primary antibody (1/25 dilution for HAS2 and 1/200 dilution for VDR) was pipeted onto the blot, covered in parafilm, and incubated in a humidity chamber at 4°C overnight.

The next day, the blots were washed three times in PBS. Next, 10mL of secondary antibody (1:2000 dilution of anti-goat-HRP or anti-mouse-HRP) was put on each blot. After, the blots were once again washed in PBS 3 times and ECl Plus (1:40) was mixed and applied to each blot under parafilm. The blots were then covered in aluminum foil and allowed to incubate for 10 minutes. After incubation, each blot was scanned on the Storm scanner.

For qPCR assays, cDNA (complimentary DNA) was prepared and analyzed as described in Section II for Has1, Has2, Has3, Hyal1, Hyal2, and Hyal3. GAPDH and 18S were used as the endogenous controls. The cDNA samples were plated in duplicate in a 384 well plate. Once plating was done, qPCR was performed using the 7900HT Fast Real-Time PCR system (Applied BioSystems). For each primer, gene expression was normalized to GAPDH and 18S expression. The data is presented as fold change in gene expression in treated samples relative to vehicle treated control samples.

c. Results

In the first series of studies we performed histological analysis of Has2 and HA in tissues from WT and VDR mice. Staining for Has2 in mammary glands of adult female WT and VDRKO mice is shown in Figure 3. In tissue from WT mice, Has2 staining is not detected. In contrast Has2 positive cells were readily detected in tissue from VDRKO mice. The staining for Has2 in VDRKO mice is localized to a subset of cells in the stroma (likely fibroblasts) which surround the ducts. Has2 staining in skin from adult WT and VDRKO mice is shown in Figure 4. In WT mice, the epidermis is thin and orderly, and the dermis contains hair follicles and sweat glands. Has2 staining was detected in small patches in the dermis, but was not detected at the interface between the dermis and the epidermis where the basal keratinocytes reside. In contrast, the skin of VDRKO was markedly abnormal, with thickened epidermis, disordered dermis and aborted hair follicles. Has2 in skin of the VDRKO mice was highly expressed in the basal keratinocyte cell layer where the dermis and epidermis meet. Staining in the VDRKO tissue was also detected in patches within the dermis at levels higher than that seen in WT mice.

WT VDRKO

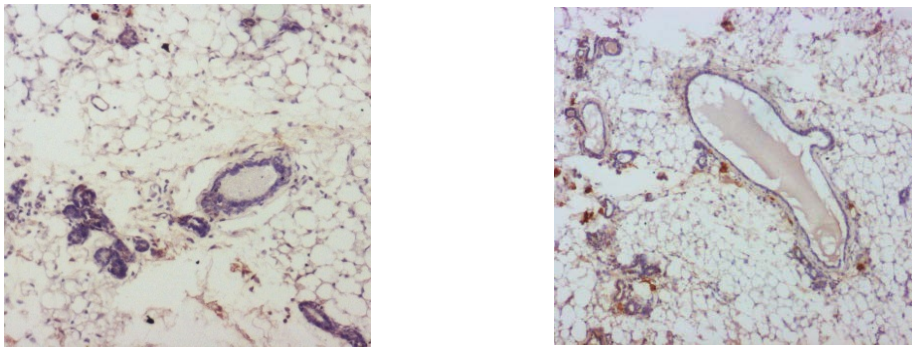

Figure 3: Has2 staining in WT and VDRKO mammary glands. The presence of Has2 was detected with Has2-specific antibody and DAB staining (brown). Sections were counterstained with hematoxylin to visualize stroma and d are representative sections of mamma WT from virgin adult female WT and VDRKO ce.

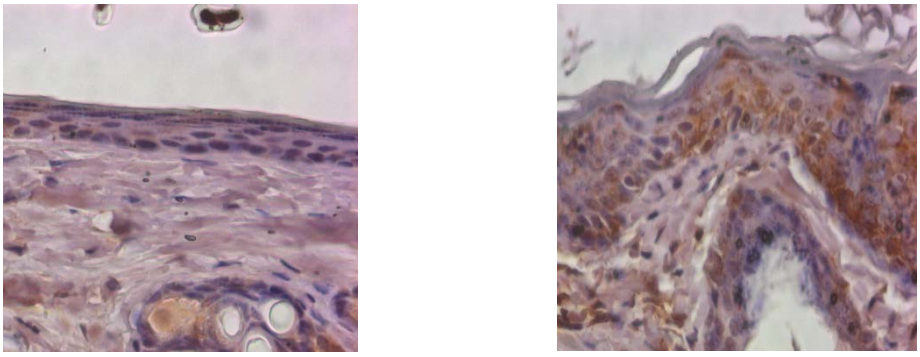

Figure 4: Has2 staining in WT and VDRKO skin tissue. The presence of Has2 was detected with Has2-specific antibody and DAB staining (brown). Sections were counterstained with hematoxylin to visualize epidermis and dermis. Shown are representative sections of skin from adult WT and VDRKO mice.

To determine whether the amount of Has2 detected in WT and VDRKO skin correlated with the amount of HA, we used a biotinylated HA binding protein (HABP) to localize HA in tissue. As shown in Figure 3, low levels of HA were present surrounding the mammary ducts of WT mice, but much larger patches were observed in tissue from VDRKO mice. A similar trend was seen in mammary tumors removed from WT and VDRKO mice with low abundance of HA in WT tumors and higher staining in VDRKO tumors. Figure 4 shows HA staining in WT and VDRKO skin tissue. Similar to the Has2 staining, HA was barely detectable in tissue from WT mice and was confined to the dermis adjacent to hair follicles and sweat glands. In contrast, the area surrounding the basal keratinocytes at the interface between the dermis and the epidermis was heavily stained in tissue from VDRKO mice. In a final set of experiments we stained mammary tissue and tumors from WT and VDRKO mice with alcian blue, a general stain for mucins which detects HA. As shown in Figure 5, Alcian blue staining was similar to HA staining. Bright blue alcian staining was rarely detected in normal tissue from WT mice but punctate areas were frequently seen in tissue from VDRKO mice. In tumors, alcian blue staining was elevated compared to normal tissue, and the differences between WT and VDRKO tissue was less obvious.

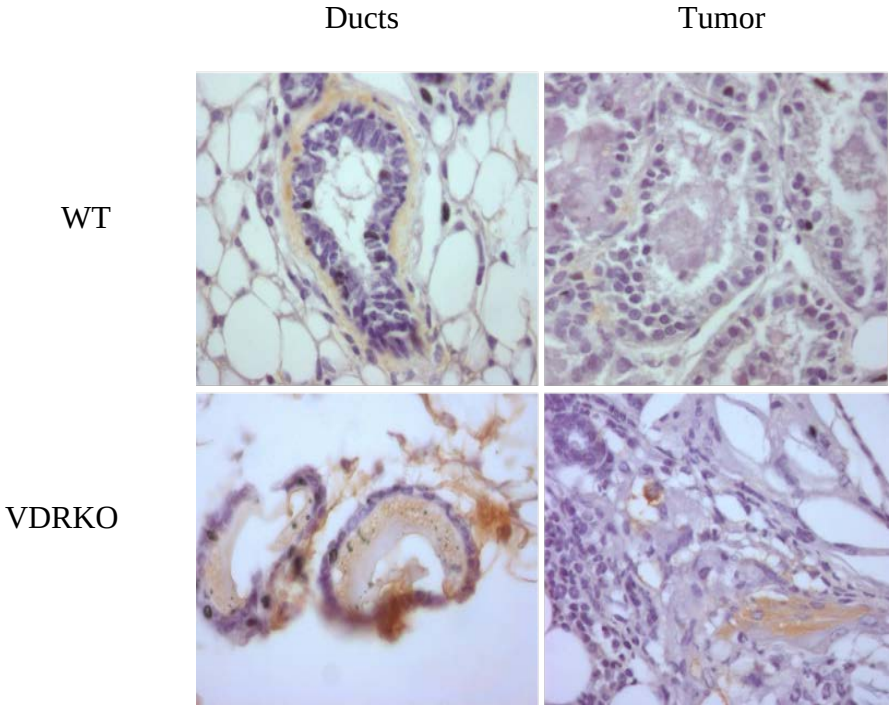

Figure 5: HA binding protein (HABP) staining for HA in WT and VDRKO mammary glands. The presence of HA was detected with biotinylated HABP and DAB (brown) staining. Shown are representative sections of ducts from normal glands and DMBA-induced tumors from WT and VDRKO mice.

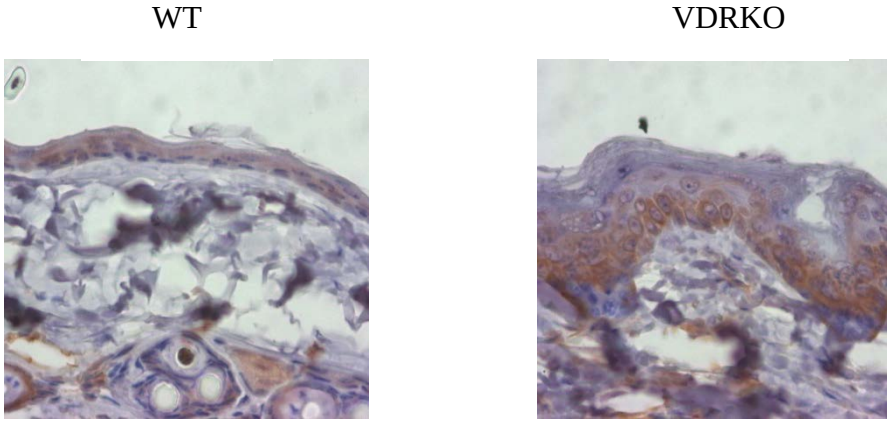

Figure 6: HA binding protein (HABP) for HA in WT and VDRKO skin samples. The presence of HA was detected with biotinylated HABP and More DAB (brown) staining staining. Shown are representative sections from normal skin removed from adult WT and VDRKO mice.

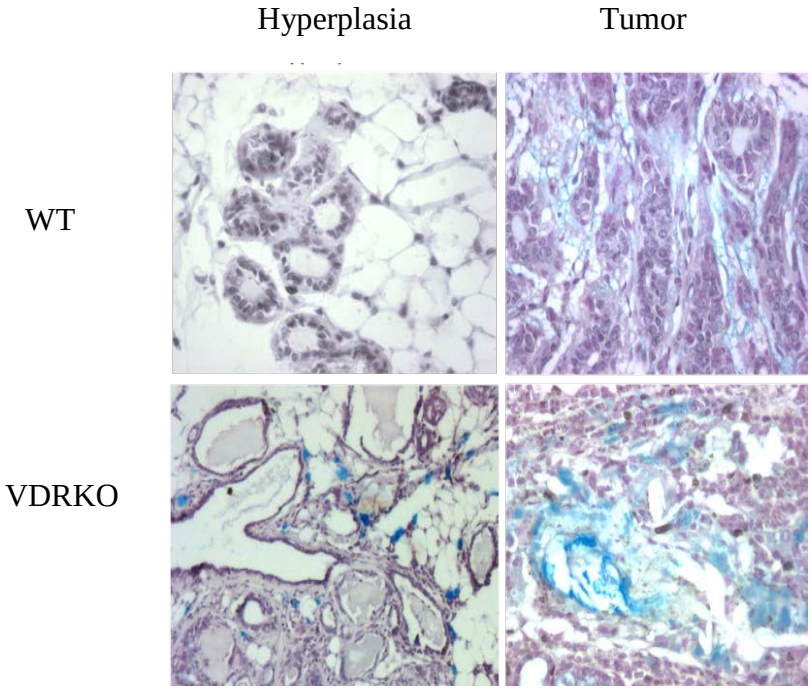

Figure 7: Alcian blue stain for proteoglycans in mammary gland hyperplasias and tumors from WT and VDRKO mice. Alcian blue staining allows for visualization of HA build-up. The increased amount of blue staining seen in the VDRKO mammary gland hyperplasias and tumors indicates HA build-up in these tissues.

In the second series of experiments on Has2, we conducted in vitro trials to measure Has2 regulation in cellular models.We cultured the mouse mammary cells with agents known to alter Has2 expression including vitamin D and all trans retinoic acid (ATRA, a form of vitamin A) and prepared cell lysates for western blotting. These experiments were designed to test whether a commercially available Has2 antibody would successfully detect murine Has2. The results are shown in Figure 8. Samples include four cell lines (KO240, WT145, KO^{hVDR4} and HC11 cells) treated with ethanol, vitamin D, ATRA, or the combination of vitamin D and ATRA. The Has2 protein is 63kDa and should run approximately in the center of a 7% SDS-PAGE gel.

Unfortunately, it is difficult to determine whether any of the detected bands correspond to Has2, as there were multiple protein bands present in the central area. An arrow is used to designate the band that most likely corresponds to the Has2 protein of 63kD. However, this band is barely detectable in these murine cell lines, and the data are inconclusive both with respect to detection of Has2 and with respect to the effects of the various treatments. Further work is needed to optimize this antibody or test additional antibodies. In Figure 9, the same samples were blotted with an antibody that detects the VDR protein. In this blot the 50kDa VDR protein (arrow) is clearly visible in lysates of WT145 and HC11 cells but not in KO^{hVDR} cells or KO240 cells. The effect of treatment with ethanol, vitamin D, ATRA, or the combination of vitamin D and ATRA appear to be most pronounced in the WT145 cells, where both vitamin D and the combination treatments up-regulated VDR expression.

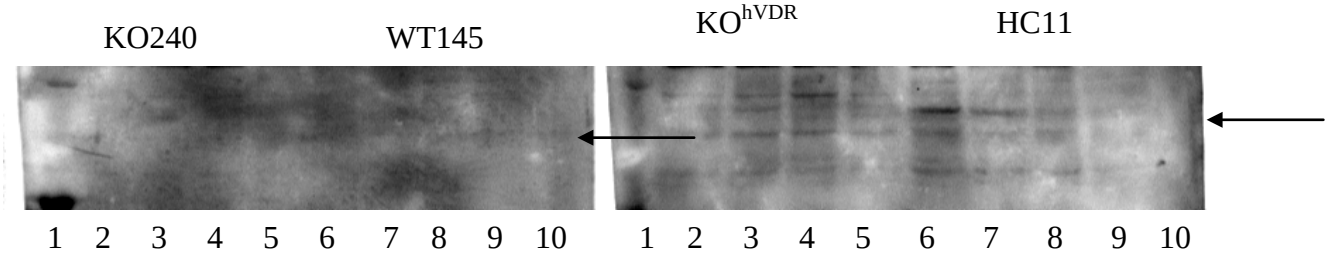

Figure 8: Western blot for Has2 in KO240, WT145, KO^{hVDR}, and HC11 cells treated with 100nM EtOH, vitamin D, ATRA, or combo. Cell lysates were separated on SDS-PAGE and blotted with antibody that recognizes Has2. Arrow indicates expected location of Has2 protein (63kDa).

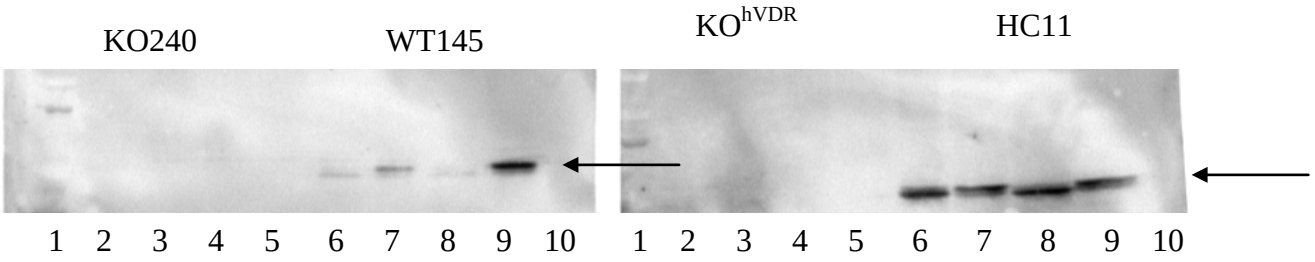

Figure 9: Western blot for VDR in KO240, WT145, KO^{hVDR}, and HC11 cells treated with 100nM EtOH, vitamin D, ATRA, or combo. Cell lysates were separated on SDS-PAGE and blotted with antibody that recognizes human and mouse VDR. An immunoreactive band at 50kDa represents VDR and is indicated by the arrows.

We also conducted qPCR studies in the KO^{hVDR} and HC11 cell lines to assess expression of Has and Hyal genes after these treatments. The data are summarized in Figure 10. Based on the microarray screening and other data from the Welsh lab, it was expected that Has2 would decrease with vitamin D treatment—however, we found no change in the expression of Has2 (or any of the other genes examined) in the KO^{hVDR4} cells. This may reflect the low level of VDR present in these cells (as evident in Figure 9). Interestingly, ATRA up-regulated all three Has genes (2-4 fold) and Hyal3 (2-fold). No further increases were observed in cells treated with both ATRA and vitamin D. In the HC11 cells, the only significant change with vitamin D treatment was a down regulation of Has2 (-2.8). Although as expected, Has2 was increased in KO^{hVDR} cells treated with ATRA, no effect of ATRA was observed in HC11 cells. The combination treatment in HC11 cells further increased Has2 above that detected with vitamin D alone, and enhanced the expression of Hyal1, which was up regulated to 8.8. No other changes of significance were noted.

	KO^{hVDR4}			HC11		
	VitD	ATRA	Combo	VitD	ATRA	Combo
Has1	1.9	4	4	1.5	1.6	1.2
Has2	-1.1	2.3	-1.1	-2.8	-1.5	-3.5
Has3	1.5	2.5	2.8	-1.1	-1.1	1
Hyal1	1.1	1.5	1.3	-1.1	-1.9	8.8
Hyal2	1.1	1.2	1.4	-1.3	-1	-1
Hyal3	1.7	2.3	4	-1.1	1.1	1.4
hVDR	-1.2	1.5	1.1	N/A	N/A	N/A

Figure 10: Expression of Has and Hyal genes in KO^{hVDR} and HC11 cells. Cells were treated with ethanol vehicle, 100nM 1,25D, 100nM ATRA or both 1,25D and ATRA for 24 hours. RNA was isolated and used for qPCR with gene-specific primers and GAPHDH for normalization. Data are expressed as fold change of vehicle treated cells.

d. Discussion

Based on previous data from the Welsh laboratory showing that vitamin D suppresses Has2 gene expression *in vitro*, we further examined the regulation of hyaluronan metabolism in cell and mouse models. We hypothesized that if vitamin D is a physiologic regulator of Has2, then both Has2 and HA would be increased in tissues from VDRKO mice. Through histological approaches we visualized Has2 and HA in normal tissue and tumors derived from skin and mammary gland of WT and VDRKO mice. Our data strongly suggests that Has2 is elevated in VDRKO tissue, most dramatically in the skin. The up-regulation of Has2 in both skin and mammary gland was correlated with increased abundance of HA as detected with HABP and Alcian blue staining. Collectively, these data indicate that tissues lacking functional VDR

exhibit increased Has2 and HA which col-localize. Thus, these data support our hypothesis that VDR deficiency would result in up-regulation of Has2 .

One implication of our findings is that suppression of Has2 and HA by vitamin D contributes to the control of cell proliferation and differentiation in the mammary gland and skin. These two tissues are highly susceptible to carcinogenesis in the absence of the VDR (Zinser et al, 2002; 2005). Because HA exerts tumor promoting actions, it is possible that the suppression of Has2 and HA by vitamin D may explain why VDRKO mice have increased susceptibility to skin cancer. In addition, it is possible that the excess Has2 contributes to the abnormal skin phenotype in the VDRKO mice, since Has2 has recently been linked to the wrinkly skin phenotype of Shar-Pei dogs (Olsson et al, 2011). The final implication of our findings is the potential relevance of vitamin D to human skin cancer. The VDRKO mice are an accurate model for a human genetic condition characterized by VDR mutations which render the VDR non-functional. In this condition, the development of the hair follicle in the skin is disturbed, resulting in the same bald phenotype as observed in the VDRKO mice. Thus, the abnormalities in the VDRKO skin, including the accumulation of HA, may shed light onto the role of VDR in human skin and hair function. Since the VDR exerts similar functions in mouse and human mammary glands and skin, optimal vitamin D status may exert protection against human breast and skin cancer, as has been suggested by epidemiological studies.

With respect to the final cell studies on Has2 expression and vitamin D, further work is needed to validate our findings. The western blot for Has2 showed multiple bands and is therefore inconclusive as it is difficult to locate the specific protein band that corresponds to Has2. To fix this problem, additional blots should be conducted to optimize the antibody staining for detection

of Has2. Once optimized it will be necessary to normalize and quantitate Has2 expression between different treatments. The VDR blot must also be repeated because no VDR was detected in the KO^{hVDR4} cells. This might reflect insufficient protein loading in these lanes or a change in the cell line itself that caused loss of the ectopic VDR. Furthermore, it is necessary to repeat all these experiments since not enough replicates were performed to ascertain the reproducibility of the results. It will be especially important to repeat the PCR and western blot experiments to conclusively demonstrate the link between vitamin D, the VDR and Has2 in relation to tumorigenesis.

IV. OVERALL CONCLUSIONS

In this thesis I conducted laboratory research focused on the molecular actions of vitamin D especially in relation to protection against chronic diseases such as cancer. Although the initial cytokine studies were conducted in normal cells, they support a role for vitamin D in regulation of inflammation, which is intimately involved with cancer progression. Our data provides evidence that the anti-inflammatory effects of IL10 are increased in the presence of vitamin D. Importantly, we also observed differences in the effects of vitamin D on cytokines in normal mammary cells versus breast cancer cells. The loss of vitamin D control over immune gene regulation may be an important contributor to cancer initiation or progression in the mammary gland. We also provide evidence that VDR, the receptor for active forms of vitamin D, exerts anti-cancer effects in skin and mammary tissue through the suppression of Has2. The suppression of Has2 by vitamin D and the VDR would be expected to inhibit the cancer process because high levels of Has2 and its product HA are known to promote cell proliferation and cancer. Although these experiments were performed using cell and mouse models, epidemiological studies have consistently linked low vitamin D status with increased risk for cancer in many tissues. Thus, further studies are warranted to mechanistically investigate whether regulation of cytokines or HA production by vitamin D in the context of human cancer.

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