# The Role of Tumoral 1,25 Dihydroxyvitamin $D_3$ in Inhibition of Tumor Growth and Progression in the PyVMT MMTV# 634 Transgenic Breast Cancer Model

By

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# Abstract

Vitamin $D_3$ must be metabolically activated by the liver to 25-hydroxyvitamin $D_3$ ($25OHD_3$) and then by the kidney 1αhydroxylase (1αOHase) to become 1,25dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$). $1,25(OH)_2$ $D_3$ is a potent inhibitor of tumor growth *in vitro* and *in vivo*. Recent studies indicate that metabolic activation of $1,25(OH)_2D_3$ also occurs in cancer cells such as breast cancer. Consequently, the major objective of this project was to determine if tumoral $25OHD_3$-1αhydroxylase modulates any or all of the stages of breast tumor progression without inducing the hypercalcemic side effects of $1,25(OH)_2D_3$. For this purpose we used the PyVMT breast cancer mouse model in which the oncoprotein, polyomamiddle T antigen (PyMT) is under the control of mouse mammary tumor virus LTR (MMTV LTR). Mice exhibited tumors restricted to the mammary epithelium progressing to the various stages of breast cancer. Animals were treated with either vehicle, $25OHD_3$ (2000 pM/24h) or $1,25(OH)_2D_3$ (12pM/24h). Mice treated with the vitamin D precursor, $25OHD_3$, exhibited a marked reduction in tumor onset and growth comparable to the $1,25(OH)_2D_3$ treated group. Furthermore biomarkers of tumor progression were markedly reduced in $25OHD_3$ and $1,25(OH)_2D_3$ animals as compared to vehicle-treated animals. However, mean circulating calcium concentrations remained unchanged in $25OHD_3$ treated animals but increased significantly in $1,25(OH)_2D_3$ treated animals as compared to controls. Tumoral levels of $1,25(OH)_2D_3$ in mice treated with $25OHD_3$ were increased 79% in comparison to vehicle control mice. Additionally, $25OHD_3$ and 1,25(OH)2D3 treated animals had a significant decrease in the mean number of lung metastases per animal as compared to vehicle treated control animals. This study therefore suggests an important autocrine role of 1αOHase expression in breast tumor cells. Furthermore, accumulation of intra-tumoral $1,25(OH)_2D_3$ in response to $25OHD_3$ administration strongly suggests that locally produced $1,25(OH)_2D_3$ plays a significant role in restraining tumor growth without inducing the hypercalcemic side effects associated with $1,25(OH)_2D_3$.

# Résumé

Pour acquérir son activité biologique, la vitamine $D_3$ doit subir deux hydroxylations successives, la première par le foie sur le carbone 25, la seconde par la 1-α-hydroxylase (CYP27B1) rénale sur le carbone 1 aboutissant alors à la forme active de la vitamine D: la 1,25dihydroxyvitamine

$D_3$ (1,25-$(OH)_2$-$D_3$). Le 1,25-$(OH)_2$-$D_3$ est un inhibiteur potent de la croissance tumorale aussi bien *in vivo* qu'*in vitro*. Des études récentes suggèrent aussi que l'activation métabolique de la vitamine D se produirait aussi dans des cellules tumorales comme celles du cancer du sein. Conséquemment, l'objectif principal de ce mémoire est de déterminer si la 1-α-hydroxylase exprimée par les cellules tumorales module certains ou même tous les stades de progression du cancer du sein sans toutefois induire les effets secondaires d'hypercalcacémie causés par le 1,25-$(OH)_2$-$D_3$. Le modèle animal utilisé est la souris PyVMT ("polyomamiddle T antigen"), un modèle expérimental de cancer du sein. En exprimant une oncoprotéine, l'antigène moyen T du polyome, sous le contrôle du virus de tumeur murine LTR (MMTV LTR), ces souris développent des tumeurs restreintes à l'épithélium mammaire progessant à travers les différents stades de cancer du sein. Ces animaux furent traités soit avec véhicule, 25(OH)-$D_3$ (2000 pmol/24 h) ou 1,25-$(OH)_2$-$D_3$ (12 pmol/24 h). Les animaux ayant recu le précurseur de la vitamin D ont exhibé une reduction marquée de la croissance et la taille de leurs tumeurs. De plus, l'analyse de biomarqueurs de croissance tumorale chez ces animaux a demontré que leur stade de progression tumorale était inférieur à celui des animaux traités avec véhicule. Les souris traitées avec le 25(OH)-$D_3$ ont aussi demontré une augmentation significative

(53,8 ± 0,83%) de leur niveau de 1,25-$(OH)_2$-$D_3$ comparativement aux animaux contrôles. Nous avons aussi observé, chez les animaux traités avec le 25(OH)-$D_3$, une réduction du nombre moyen de métastases aux poumons comparativement à celui des animaux traités avec véhicule. De plus, la concentration circulante de calcium est demeurée inchangée chez les animaux traités avec le 25(OH)-$D_3$ mais, celle-ci a augmenté de façon significative chez les animaux traités avec le 1,25(OH)-$D_3$ comparativement aux souris contrôles. Cette étude suggère donc que la 1-α-hydroxylase du sein aurait un rôle autocrine important dans la progression tumorale. De plus, l'accumulation intra-tumorale de 1,25-$(OH)_2$-$D_3$, en réponse à l'administration du 25-(OH)-$D_3$, suggère fortement que la production locale de 1,25-$(OH)_2$-$D_3$ joue un rôle significatif en restreignant la croissance tumorale et ce, de facon autocrine, sans pour autant induire les effets secondaires de l'hypercalcémie associés avec le 1,25-$(OH)_2$-$D_3$.

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# LIST of ABBREVIATIONS

| | |
|---|---|
| 1, 25$(OH)_2D_3$/vitamin $D_3$ | 1,25dihydroxyvitamin $D_3$ |
| 1αOHase/CYP27B1 | 25 hydroyvitaminD-1-alpha hydroxylase |
| 25-OHase | 25-hydroxylase |
| 7DHC | 7-dehydrocholesterol |
| AFP | α fetoprotein |
| ALB | Albumin |
| C cells | Parafollicular cells |
| $Ca^{2++}$ | Calcium |
| CaR | Calcium Sensing Receptor |
| CDK | Cycle dependent kinases |
| CDKI | Cycle dependent Kinases inhibitors |
| CRH | Cytokine receptor homology |
| DBP | Vitamin D binding protein |
| DR | Direct repeat |
| ECD | Extra cellular domain |
| FVB | *Fv1*$^b$ allele for sensitivity to the B strain of Friend leukaemia inbred genetic background. |
| FGF23 | Fibroblast growth factor 23 |
| GPCR | Guanine nucleotide regulatory coupled receptor |
| HDAC | Histone deacetylases transferase activity |
| HL-60 | Promyeloctic leukemia cells |
| HPV | Human Papilloma virus |
| HRE | Hormone response elements |
| IHC | Immunohistochemistry |
| IkB | Inhibitor protein I-kB |
| IL-1 | Interleukin-1 |
| IP3/DAG | Inositol 1,4,5 trisphosphate and diacylglycerol |
| JAK | Janus family protein tyrosine kinase |
| LBD | Ligand binding domain |
| LTBP Latent | TGF-ß Binding Protein |
| LTR | Long terminal repeats |
| m1 | Myeloid leukemia cells |
| MAPK | Mitogen-activated protein kinase |
| MEPE | Matirx extracellular phosphoglycoprotein |
| MMTV | Mouse mammary tumor virus |
| NCoR | Nuclear corepressor |
| NF-kB | Nuclear factor kappa β signaling |
| PAI-1 | Plasminogen activator inhibitor |
| PHEX | Phosphate-regulating endopeptidase homologue X linked gene |
| PI3K | Phosphatidylinositol-3phosphate kinase |
| PTH | Parathyroid Hormone |
| PyV | Polyomavirus |
| RANK | Receptor activator of NF-κB (RANK) |
| RANKL | Receptor activator of NF-κB ligand |

| | |
|---|---|
| RAS | Ras is the name of a protein, the gene that encodes it, and the family and superfamily of proteins to which it belongs. |
| Rb | Retinoblastoma |
| RRSBP | Rapid response steroid binding protein |
| RTK | Receptor tyrosine kinases |
| RXR | Retinoid X receptor |
| SHIP | Ski-Interacting Protein |
| SMRT | Silencing mediator for retinoic and thyroid hormone |
| SOCS | Suppressor of cytokine signaling proteins |
| SRC | Steroid receptor coactivators |
| SRF | Serum response factor |
| STAT | Signal transducers and coactivators of transcription |
| TBP | TATA binding protein |
| TCF | Ternary complex factor |
| TGF β | Transforming growth factor beta |
| TNFα | Tumor necrosis factor alpha |
| UVB | UltraViolet B |
| VDDR Tvpe 1 | Vitamin dependent rickets Type 1 |
| VDDR type 2 | Vitamin dependent rickets Type 2 |
| VDR | Vitamin D receptor |
| VDRE | Vitamin D response elements |
| XLH | Familial Hypophosphatemic Rickets |

# Chapter 1 Introduction

This thesis will start with an overview of general concepts concerning vitamin D and Cancer. Calcium homeostasis and molecular mechanisms of vitamin D interaction with signaling cascades relevant to the mechanism of tumor progression will be described. The concept of vitamin $D_3$ as a therapeutic agent against cancer will be explored. Work accomplished in our laboratory and in this area of research will be discussed. Finally, the objectives and preliminary results of this research project will be described.

## 1.1 Vitamin D in History

The first observation of the effects of vitamin D in the human system was described in the early 17$^{th}$ century (1). A new undocumented disease appeared in Europe and was first reported in London England by Daniel Whistler and successively by Boote, Glison, and Mayow (1,2). This disease was called "rickets" and resulted in large number of deaths throughout the European continent. The exact origin of the word rickets still remains unknown today (2,3). Rickets was characterized as bone deforming disease that resulted in growth retardation, enlargement of epiphyses of the long bones, deformities of the legs (Figure 1A), bending of the spine and weak toneless muscle in children living in the densely populated areas of London (Figure 1B,1C)(4, 5). As Europe entered the industrialization age, the use of coal became widely prevalent resulting in darkening of cities by smog which progressively blocked and decreased the amount of sunlight that residents of these metropolises would receive (6).Until this point, no known effective treatment for rickets was being employed by physicians. In 1822 a polish physician observed the importance of sun exposure for the treatment, cure and prevention of rickets.


**Figure 1: Skeletal Deformities Observed in Rickets. (A)** Photograph from the 1930's of three children .The child in the middle is not affected by rickets while the two girls on the left and right portray classic knock knees and bow legs, growth retardation, and other skeletal deformities. **(B)** Radiograph of the radius and ulnae from a rickets stricken child. **(C)**Radiograph of a child's radius and ulnae affected by rickets but exposed to UVB treatment (24).

Sunbathing became the revolutionary mode of treatment for this new disease (7). In the early 20th century experimentation utilizing radiation from quartz lamps exposing children three times a week was seen as a more effective treatment in comparison to sunbathing. Dr. Huldschinski concluded that UV radiation was an successful treatment for rickets (Figure 1B,C)(8). Following this discovery, cod liver oil was then identified as a nutritional source that prevented rickets and resulted in the mistaken identification of vitamin $D_3$ as a vitamin (9). Upon this discovery, Vitamin $D_3$ was then structurally identified, chemically synthesized and subsequently its precursor 7-dehydrocholesterol (7DHC) was identified in the skin (4,5,10). Thus, it took almost three centuries to determine that rickets was a disease that resulted from an insufficient supply of vitaminD.

## 1.2 Global Effects of Vitamin D Deficiency on Calcium and Skeletal Homeostasis

25OHD is produced in the liver as a precursor of 1,25dihydroxyvitamin D (10). Optimal circulating concentrations of 25OHD is still debated but is assumed to be in the range of 50-90ng/mL (Table1)(11,12). Table 1 summarizes different $25OHD_3$ circulating concentrations and their health implications. In young children and infants vitamin D deficiency disrupts chondrocyte maturation and stops the mineralization of growth plates causing widening of epiphyseal plates. Furthermore, the skeleton is poorly mineralized due low calcium and phosphorus product resulting in an unstable skeleton (Figure 1A). Thus, when a vitamin D deficient child begins to stand, gravity causes the bowing or concavity of the long bones in the legs (13,14). In adults, severe vitamin D

| 25(OH)D (ng/mL) | 25(OH)D (nmol/L) | Health Implications |
|---|---|---|
| <20 | <50 | Deficiency |
| 20-32 | 50-80 | Insufficiency |
| 32-100 | 80-250 | Sufficiency |
| 54-90 | 135-225 | Normal In Sunny countries |
| >100 | >250 | Excess |
| >150 | >325 | Intoxication |

**Table 1: Health Implications Associated with Circulating Concentrations of 25OHD.** Vitamin D deficiency results in a negative calcium and skeletal balance due to decreased intestinal absorption of dietary calcium and phosphorus. Vitamin D deficiency prevents efficient absorption of calcium in the intestine and calcium reabsorption in the kidney. Levels below 20 ng/ml result in vitamin D deficiency and have many health consequences (11).

deficiency results in osteomalacia (25,26) characterized by secondary hyperparathyroidism and a decrease in the mineral content of the bone matrix (uncalceified osteoid) of the skeleton; moreover secondary hyperparathyroidism causes an increase in phosphorus wasting in the kidney (phosphaturia) (11).

The primary result of vitamin D deficiency is a negative calcium and skeletal balance due to decreased intestinal absorption of dietary calcium and phosphorus (Figure 2) (15,16). Vitamin D deficiency prevents efficient absorption of calcium in the intestine and calcium reabsorption in the kidney (4). The transient decrease in ionized calcium stimulates the parathyroid glands to secret parathyroid hormone (PTH) in an attempt to correct this decrease by acting on the renal system to increase renal calcium tubular reabsorption in the distal convoluted tubules and to stimulate $1,25(OH)_2D_3$ production (17,18). PTH also stimulates calcium resorption from bone by interacting with its membrane receptor on mature osteoblasts, which induces the expression of receptor activator of NF-κB ligand (RANKL) (19,20). RANKL is recognized by receptor activator of NF-κB (RANK), which is present on the plasma membrane of pre-osteoclasts. Interaction between RANKL and RANK results in increased production and maturation of osteoclasts. The osteoclasts release hydrochloric acid & collagenases to destroy bone, resulting in the mobilization of the calcium stores out of the skeleton trying to correct for the decrease in circulating ion calcium concentration (Figure 3) (19,20,21). Thus, vitamin D deficiency-induced secondary hyperparathyroidism results in the skeletal resorption which together with osteomalacia results in osteoporomalacia (25,26).

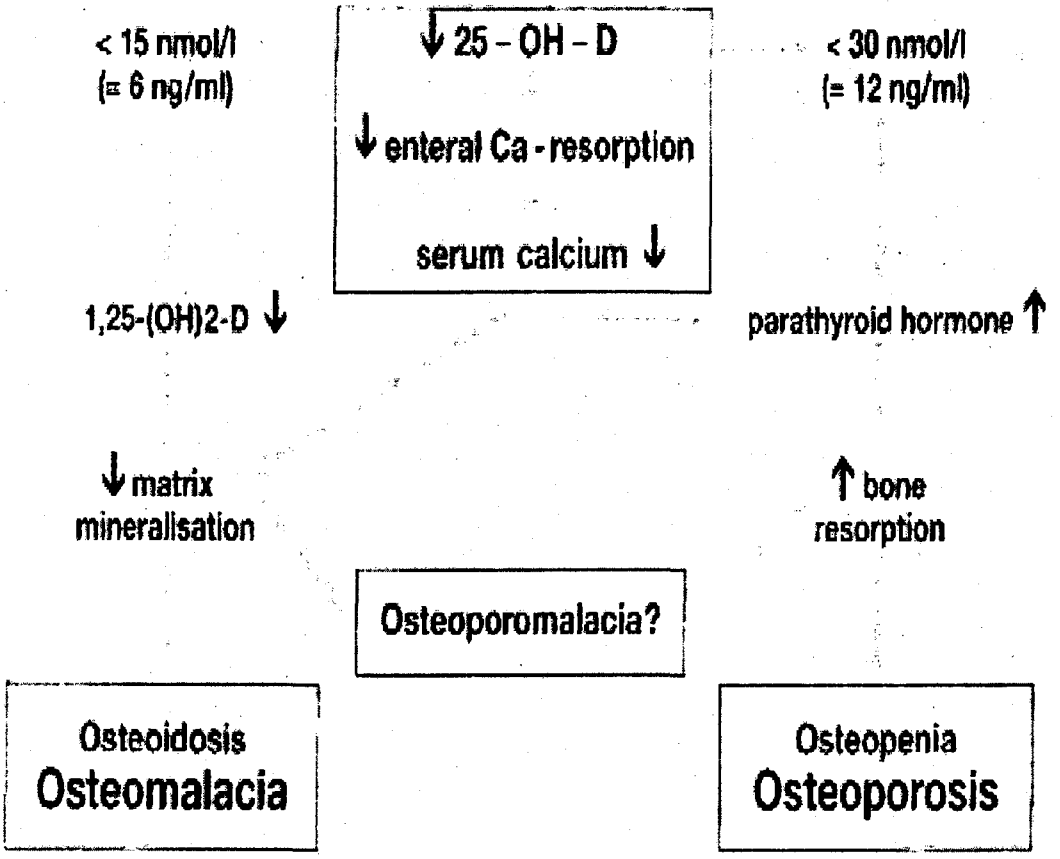



**Figure 2: Effects of Decreased vitamin D Concentration on Calcium and Skeletal Homeostasis.** The circulating blood concentration levels of the vitamin D precursor 25OHD are strictly regulated. Effects of Long term vitamin D deficiency cause decreased matrix mineralization, increased bone resorption and an increase in the secretion of the Parathyroid hormone. This can ultimately result in osteomalacia, osteoporosis or a state of combined diseased with a potential risk of secondary hyperthyroidism (24).

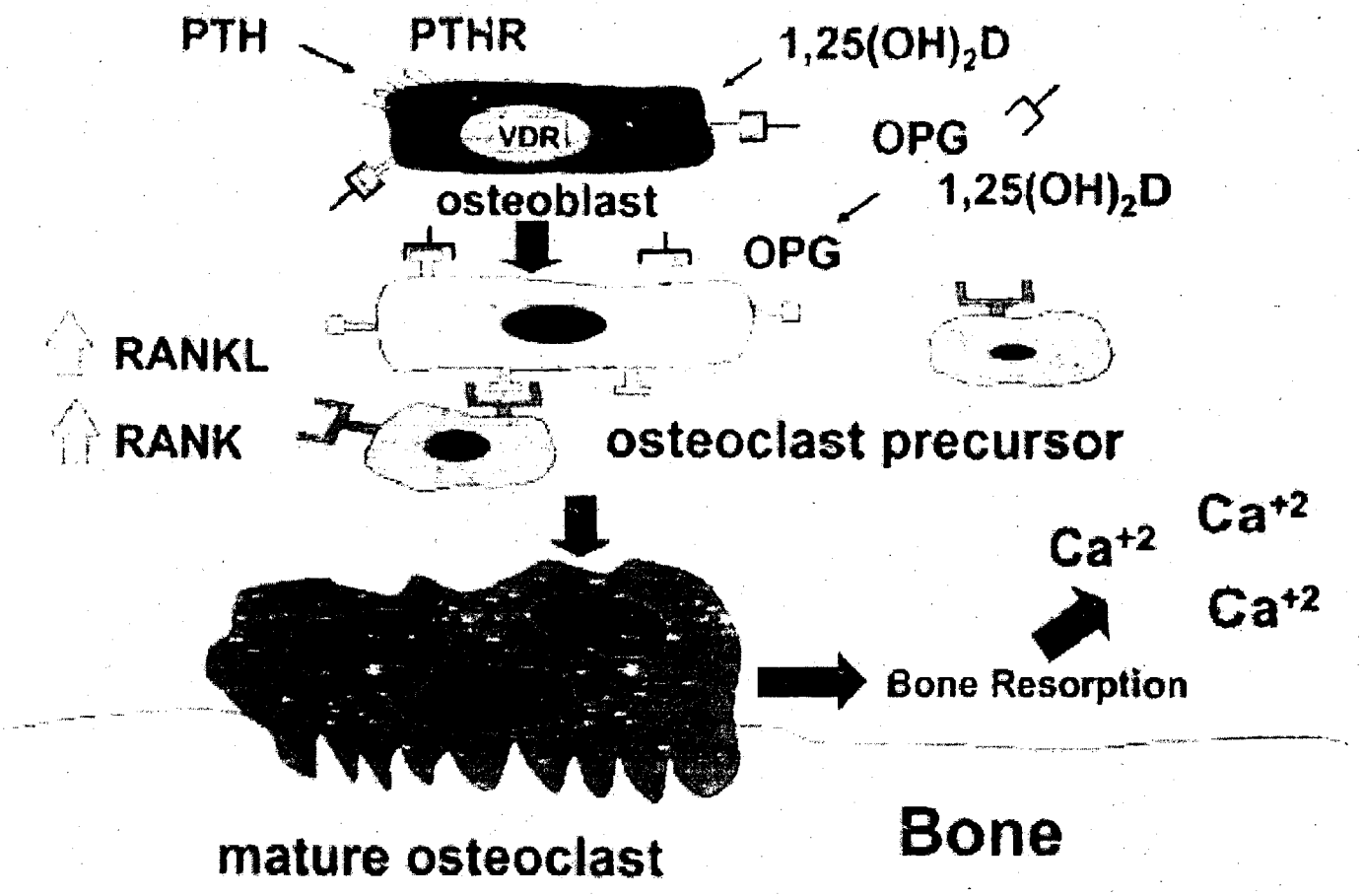



**Figure 3: Mechanism of Bone Turnover.** Upon binding of PTH to the PTH receptor (PTHR) or Vitamin D to the vitamin D receptor on osteoblasts; the RANK/RANKL system modulates the activation and maturation of osteoclast. Release of hydrochloric acid & collagenases occurs resulting in bone resorption (304).

## 1.2.1 The Various Forms of Rickets

Vitamin D deficiency or the lack of vitamin D effects on target cells results in rickets . The various forms of this disease will now be discussed in detail.

## 1.2.2Vitamin D Deficient Rickets

Vitamin D deficient Rickets is the result of inadequate supply or synthesis of $1,25(OH)_2D$ in the body (11). The various stages of vitamin D deficiency associated with 25OHD levels are summarized in table1. There are numerous factors that play central roles in vitamin D deficiency including: living in high altitudes, clothing that cover the skin surface area, skin pigmentation (melanin absorbs UVB radiation diminishing cutaneous vitamin $D_3$ synthesis) and the lack of dietary intake of sufficient amounts of vitamin D (22,23). Due to the inadequate amount of vitamin D in the diet, normal dietary intake cannot provide the required levels of vitamin D (7 to10µg/day (280- 400 IU)) needed to prevent rickets (11,24).Vitamin D deficiency is diagnosed by measuring circulating levels of its metabolite 25OHD which is the most abundant vitamin D metabolite that represents the vitamin D status of an individual. Levels below 30 nmol/l are associated with stage I vitamin D deficiency while levels below 17nmol/l are associated with osteomalacia or stage III of vitamin D deficiency. Decreased circulating levels of 25OHD result in various skeletal abnormalities alone or in combination including osteoporosis, osteopenia, osteoidosis, osteomalacia and osteoporomalacia (4,11,15,24). However, more recent studies indicate that levels below 75nmol/L represent a state of "vitamin D insufficiency"(11).

## 1.2.3 Osteomalacia/Adult Rickets

In the adult, vitamin D deficiency results in inadequate mineralization of the osteoid matrix known as Osteomalacia/Adult Rickets (25,26). The osteoid is the bone protein matrix, composed primarily of type 1 collagen. When there is insufficient mineral or osteoblast dysfunction, the osteoid tissue does not mineralize properly and accumulates resulting in the "Looser's" zone or pseudofractures (Milkman's fractures) (25, 26). Osteomalacia is characterized by non-specific generalized bone pain and increased risk of bone deformities and fractures.

## 1.2.4 Pseudovitamin D Deficiency Rickets (Type1)

PseudovitaminD-Deficiency Rickets is an inherited autosomal recessive condition. It is characterized by rickets, hypocalcemia, tooth enamel hypoplasia, secondary hyperparathyroidism, hypotonia, weakness, growth failure and reduced serum concentration of $1,25(OH)_2D$ in the face of normal concentrations of 25OHD (27,28). It is also known as Vitamin dependent rickets Type 1 (VDDR type 1). In this disease, 25-hydroxyvitamin D-1α hydroxlase which converts 25 hydroxyvitamin D to 1 alpha, 25 - dihydroxyvitamin D is defective. It is due to inactivating mutations located on the gene coding for the P450c1 component of the renal 1 alpha-hydroxlase, located on chromosome 12q13.1-13.3 (27). Mutations have been identified, including several missense; exon 2 Arg107His, exon2 Gly125Glu, Exon 6 Arg 335Pro and exon 7 Pro382 Ser (29). Administration of $1,25(OH)_2 D_3$ to these patients correct all the abnormalities observed (30).

## 1.2.5 Hereditary Vitamin D Resistant Rickets (Type2)

Hereditary vitamin D resistant rickets is an inherited autosomal recessive condition cause by target organ resistance to the action of $1,25(OH)_2$ D. This condition is also known as vitamin dependent rickets type 2 (VDDR type 2).The disease is the result of inherited defects in the vitamin D receptor (VDR) gene located on chromosome 12q, the same region as the gene encoding for 1 alpha hydroxylase (31). Several mutations in the ligand binding domain (LBD) of the VDR as well as the DNA binding domain of the VDR have been reported (32). Mutations in the DNA binding domain create a premature stop signal that affecting normal DNA binding (32). Mutations affecting the LBD include missense mutations that result in a fully translated VDR (31,33). This form of vitamin D deficiency is characterized by rickets, hypocalcemia and in some cases; alopecia. The disease can be corrected by the administration of calcium by the intravenous route to bypass the intestinal defect and to provide adequate mineral to the bone-forming sites (30, 33).

## 1.2.6 Familial Hypophosphatemic Rickets (XLH)

Familial Hypophosphatemic Rickets was first thought to be a selective disorder affecting transepithelial transport of orthophosphate (Pi) in the kidneys and the intestines (34-36).However, it was determined that this disease is an X-linked dominant inherited disease characterized by mutations in the phosphate-regulating endopeptidases homologue X linked gene (PHEX) (34).The gene is located on the Xp22.1 position on the short arm of the X-chromosome (35). XLH is characterized by decrease absorption of calcium and phosphorus by the renal tubule and gastrointestinal tract due to the mutated

PHEX product which fails to degrade the phosphatonin which accumulates in circulation thereby acting on the kidneys and intestinal tract inhibiting re-absorption of filtered phosphate resulting in excessive renal phosphate wastage and chronic hypophosphatemia. (36,37). The end result is rickets. Inappropriately, normal as well as reduced serum concentrations of $1,25(OH)_2D$ characterize this disease (36). The disease in infants and young children are characterized by *genu varum* at the time of weight bearing, short stature with disproportionate shortening of the lower limbs. Furthermore development of dental abscesses and development of premature fusions of the cranial sutures in the skull arise as well (38). Effects are reversed by treatment of oral phosphate supplements and $1,25(OH)_2$ $D_3$, yet the effects are not totally reversible and residual skeletal deformities are a result of the disease(36,39,40).

## 1.2.7 Oncogenic Osteomalacia

Oncogenic osteomalacia is a paraneoplastic syndrome in which bone and soft tissue tumors induce changes which are associated with XLH. Most tumors associated with oncogenic osteomalacia are benign and consist of mesenchymal cells or mixed connective tissue (41). Oncogenic osteomalacia is characterized by low serum phosphorus concentrations with elevation of urinary phosphate level and serum alkaline phosphatase activity. Calcium concentrations remain normal but serum concentrations of $1,25(OH)_2D$ are low to absent while levels of its precursor 25OHD are normal (42). Oncogenic osteomalacia is reminiscent of X-linked Familial Hypophosphatemic Rickets and presents with similar symptoms. The humoral factor responsible for the biochemical abnormalities which also occur in XLH are phosphatonins(43). PHEX and matrix extracellular phosphoglycoprotein (MEPE) play central roles in this disease (44). MEPE

is highly expressed in oncogenic osteomalacia and is structurally related to the group of extra cellular matrix bone tooth proteins (44). Post-translational processing of MEPE result in a protein that is abnormally large and secreted by the tumor. It is then released into the circulation where it competes with normally processed MEPE on renal and bone receptors (45). The similarities between XLH and Oncogenic Osteomalacia are due to the same circulating factors being PHEX gene mutations which lead to irregular MEPE processing. MEPE may be processed by PHEX but the exact connection between the two are still not known (46).

## 1.2.8 Secondary Hyperparathyroidism

The four parathyroid glands secrete parathyroid hormone (PTH) that controls calcium concentrations and bone metabolism (47). Primary hyperparathyroidism is characterized by high serum PTH concentrations associated with high ionized serum calcium concentrations. Primary hyperparathyroidism can be caused by a solitary parathyroid adenoma resulting from somatic or germ line mutations and accounts for 85% of cases of hyperparathyroidism (48). The remaining cases are characterized by hyperplasia of the four glands often in the context of familial primary hyperparathyrodism or multiple endocrinal neoplasia (MEN). Secondary Hyperparathyroidism on the other hand often results from vitamin D deficiency (48). Low circulating concentrations of 25OHD characterizes vitamin D deficiency and results in low circulating concentrations of $1,25(OH)_2D$ and calcium. This in turn stimulates PTH secretion from the parathyroid glands which stimulates the 1αhydroxylase in the kidney and tends to normalize circulating $1,25(OH)_2D$ concentrations (78,79). Secondary hyperparathyroidism accelerates bone turnover and results in bone loss.

## 1.3 Vitamin $D_3$ Synthesis and Metabolism

1,25dihydroxyvitamn $D_3$ is a pluripotent seco-steroid with pleiotropic actions. It is derived from a cholesterol metabolite and from a complex number of reactions that occur in the skin, liver and kidney (49).The production of vitamin $D_3$ occurs in the skin and is directly related to the concentration of 7DHC and the quality and quantity of UV radiation reaching the skin (4,5).

In order to activate vitamin $D_3$ synthesis, the skin must be exposed to ultraviolet B (UVB) radiation of wavelength ranging from 280 to 320nm. 7-dehydrocholesterol (7 DHC) absorbs the UVB radiation and is photoconverted to provitamin $D_3$ and then to previtamin$D_3$ (Figure4) (50). The highest concentration of 7DHC in the skin is found in the stratum basale and the stratum spinosum of the epidermis in which previtamin $D_3$ synthesis occurs (51). However, overexposure to UVB does not increase previtamin $D_3$ above a maximal value of 15% of the original 7 DHC concentration. Further exposure to sunlight simply causes the photoisomerization of previtamin $D_3$ to lumisterol and tachysterol (50). Figure 5 shows all major metabolites of vitamin $D_3$ and the pathways that ultimately result in the formation of 1,25-dihydroxyvitamn $D_3$.

Provitamin $D_3$ is a four member ring steroid with two conjugate double bonds in the B ring at C5 and C7 and with a side chain that identifies 7DHC (Figure 4)(52). During irridation of provitamin D with UVB light, the diene absorbs a quantum of energy that transforms it from the ground state to an excited state. The ring structure then proceeds to open at the C9/C10 position resulting in the formation of previtamin $D_3$ and the triene structure. Once formed previtamin $D_3$ exists in two conformations: cis-cis and cis-trans. The cis–cis form is the one converted to vitamin $D_3$. Further irridation results in reversible

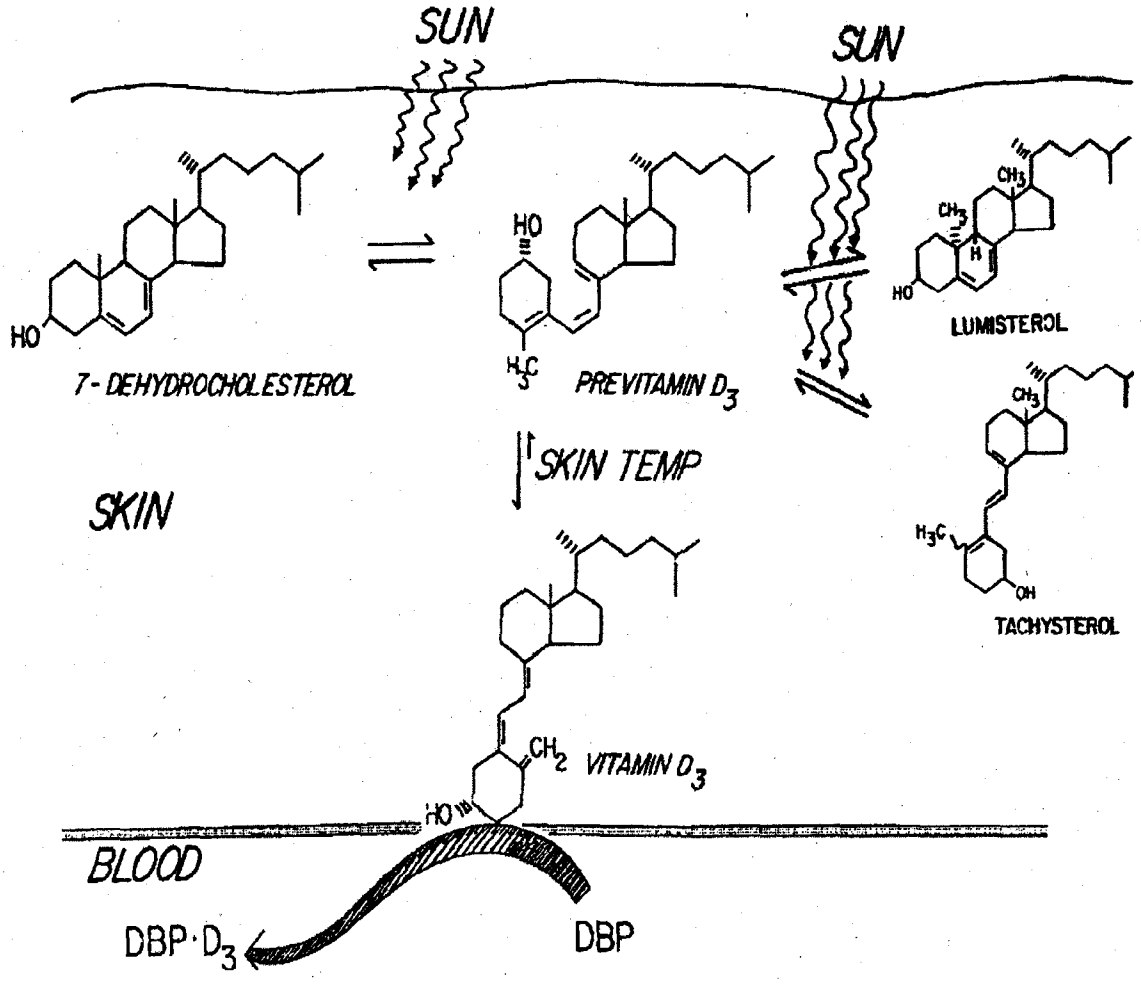



**Figure 4: Activation of Vitamin $D_3$ in the Skin.** 7DHC in the skin is activated by UVB radiation which then forms Provitamin $D_3$. Provitamin $D_3$ is a four member ring steroid with two conjugate double bonds in the B ring at C5 and C7 and with a side chain that identifies 7DHC. Upon excitation it proceeds to open at the C9/C10 position resulting in the formation of previtamin $D_3$. Previtamin $D_3$ then undergoes photoconversion to several metabolites (50).

ring closure to provitamin $D_3$ to form the stereoisomer lumisterol or isomerization to form 6,7 trans isomer tachysterol (vitamin $D_2$) (49,50,51,52)

Previtamin $D_3$, is then transported from the skin into the circulation by entering the small intestine through the lacteal system in the form of chylomicrons (53). Vitamin $D_3$ travels through the circulation by its own specific transport protein termed the vitamin D binding protein (DBP)(49,51,53).DBP then transports vitamin $D_3$ to the liver where it accumulates and undergoes hydroxylation on the C-25 by the vitamin D 25 renal hydroxylases (25-OHase)(54). In addition to the livers 25 hydroxlation of vitamin $D_3$ can also take place in the intestine and kidneys. This reaction occurs primarily in the endoplasmic recticulum and to a lesser degree in the mitochondria; yet this system is not vitamin $D_3$ specific because it carries out other cholesterol hydroxylation reactions as well (55). $25OHD_3$ is the most abundant circulating metabolite of vitamin $D_3$ (52,53,54). Subsequently, $25OHD_3$ bound to DBP enters the kidneys, binds to megalin on the plasma membrane of renal proximal tubule cells and is then transported into the cell (54). Once inside the cell, $25OHD_3$ is released and converted by mitochondrial 25 hyrxoyvitaminD-1-alpha hydroxylase (1-OHase/CYP27B1) to its active form 1,25-dihydroxyvitamin$D_3$(49-53). $1,25(OH)_2D_3$ together with PTH are regulators of calcium and phosphorus homeostasis (Figure 6).

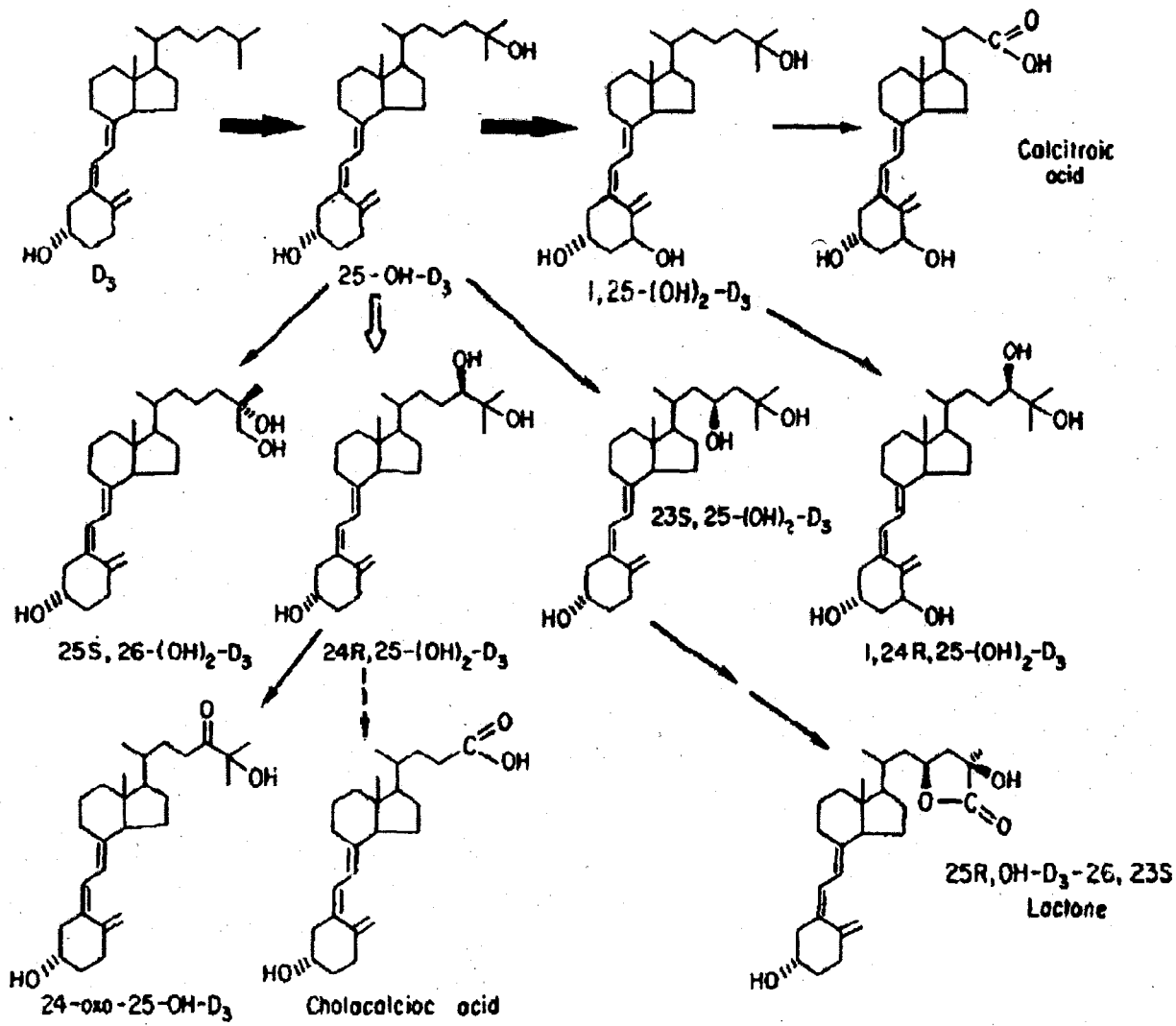



**Figure 5: Metabolic Activation and Degradation of Vitamin $D_3$.** Vitamin $D_3$ synthesis is activated by the skins absorption of ultraviolet B (UVB) radiation of wavelength ranging from 280 to 320nm. Over exposure to sunlight causes the photoisomerization of previtamin $D_3$ to lumisterol and tachysterol. Further irridation results in structural conformational changes to the overall structure of vitamin D that results in the different metabolites of vitamin $D_3$ such as vitamin $D_2$, Calcitroic acid Cholacalcioc acid and lactone (96).

## 1.3.1 1α hydroxylase (1α(OH)ase/ CYP27B1)

The kidney is the major site of metabolism of 25 hydroxyvitamin $D_3$ and the most important point of regulation of the vitamin $D_3$ endocrine system through the 1αOHase enzyme (54). In addition to the kidney, 1αhydroxylase has been located in various tissues including the liver, skin, brain, breast, testis, bone, small intestine, blood and a number of cancer cells (17,50,56).

The 1αOHase enzyme, also known as CYP27B1 belongs to the cytochrome p450 enzyme superfamily. It is located in the inner membrane of renal mitochondrial cells and requires reducing equivalents (57). Its biochemical characterization showed that CYP27B1 has a mitochondrial target signal and two conserved regions, namely the sterol-binding domain and the heme-binding domain which has a high degree of homology among p450 enzymes (58).

The renal mitochondria system is a three component system with mixed function monooxygenase and requires NADPH, molecular oxygen and magnesium ions (57). The other components are renal ferredoxin reductase, an iron sulphur protein, and cytochrome p-450 (57). The mitochondrial hydroylases are mixed function oxidases because they catalyze electron transfer from NADPH to NADPH-ferredoxin reductase through ferrodoxin (Figure 7) (59).

The kidneys are also the site of 24R-hydroxylation by 25-hydroxyvitamin $D_3$-24-hydroxylase (CYP24A1) which produce 24R25-$(OH)_2D_3$ (60). Cholecalcioic acid has been identified as a degregadation product of 24R25-$(OH)_2D_3$ resulting from this reaction (61). PTH, calcium, phosphorus and 1,25$(OH)_2D_3$ control 1alpha hydroxylase synthesis and activity (58,61). Low serum calcium levels stimulate PTH production which in turn activates 1alpha hydroxylase by acting on the promoter of the 1alpha hydroxylase gene.

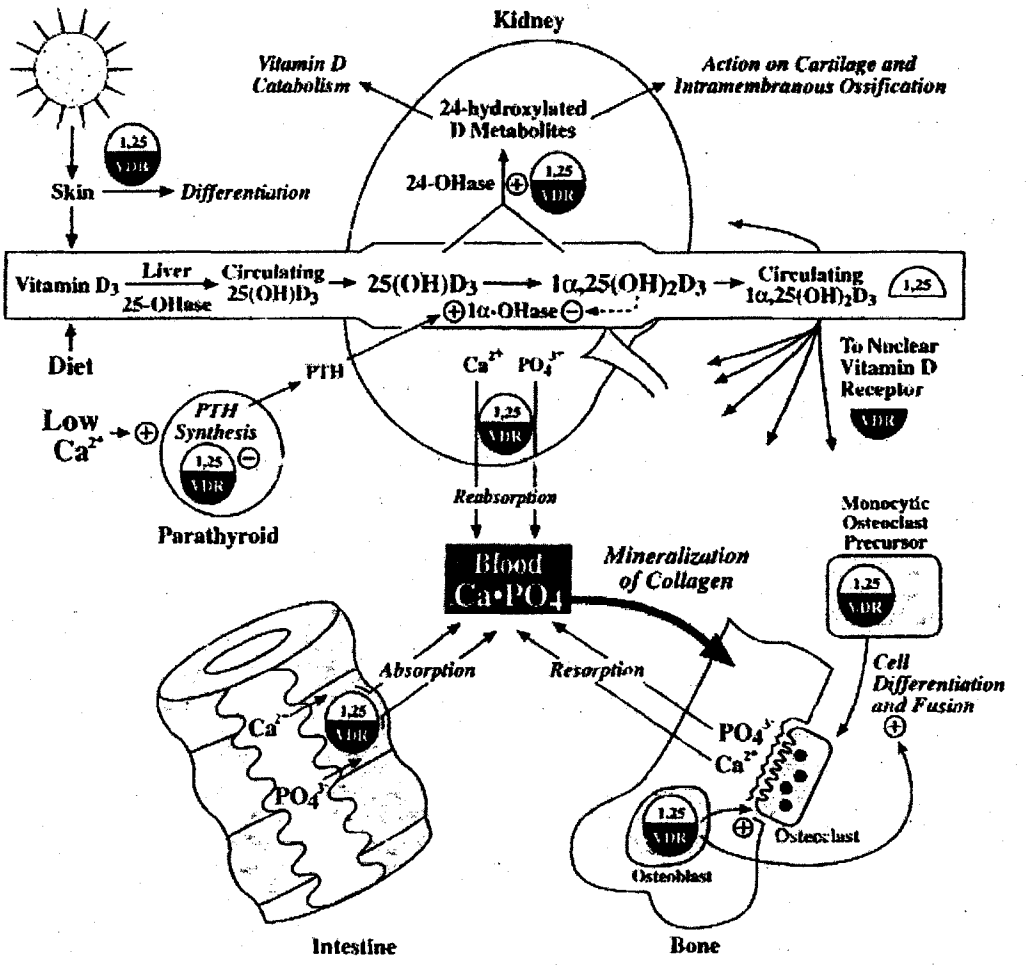



**Figure 6: 1,25-dihydroxyvitamin $D_3$ Synthesis and Metabolism.** Vitamin D synthesis is activated when the cholesterol metabolite 7DHC absorbs UVB Radiation. Following several steps of structural modification at the level of the liver and kidneys, 1,25-dihydroxyvitamin $D_3$ in conjunction with the PTH hormone regulate many key systems such as calcium and Phosphorus homeostasis. Their concentrations are under a strict negative feedback control loop which is modulated by the 1 alpha Hydroxylase enzyme and PTH / Vitamin $D_3$ circulating concentration levels (133).

$1,25(OH)_2D_3$ uses a negative feedback loop that upon sufficient serum concentration of $1,25(OH)_2D_3$ vitamin $D_3$ inhibits the 1αOHase synthesis (50,54).

As discussed in section 1.2.4, any mutation of the 1alpha hydroxylase gene results in vitamin $D_3$ deficiency (Pseudovitamin D Deficiency Rickets Type1)(27,28).In addition, mice mutants in which the 1 alpha hydroxylase has been inactivated have now been generated and have provided additional insight into the mechanisms of calcium homeostasis and rickets (58).

## 1.3.2 The Calcium Ion

Calcium ($Ca^{2++}$) is the fifth most abundant element in the biosphere and the major component of the human skeleton (62). Numerous physiological functions involve the calcium ion. The calcium ion conformation is such that it can fit into the folds of many proteins resulting in vast number of cellular proteins that have evolved to interact and bind with the calcium ion (63). Intracellular calcium is one of the main intracellular messengers and cofactors for various enzymes (64). Furthermore it is the most common signal transduction element in cells and it is this calcium transduction that regulates various cellular processes such as cell growth, fertilization, immune function hormonal secretion, muscle contraction, neural transmission and apoptosis (62,63,64). Calcium is essential for life and its concentration in the intracellular and extracellular fluids as well as in cellular and subcellular membranes must be maintained and tightly regulated (62,63,64). The main reservoir of calcium in the body is the skeleton where approximately 98% of the total body content of calcium is stored as hydroxyapatite (62).

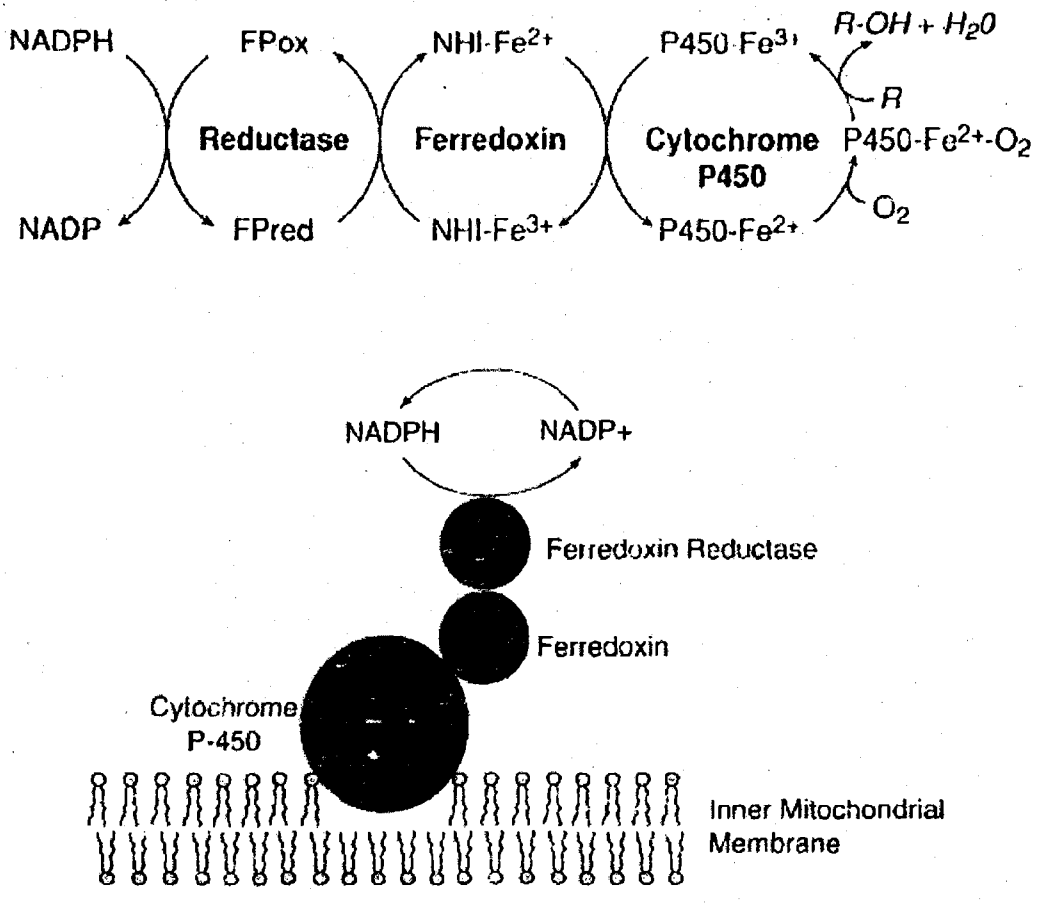



**Figure 7: Electron Transport Chain of Hydroxylases.** The 1αOHase enzyme, also known as CYP27B1 belongs to the cytochrome p450 enzyme superfamily. It is located in the inner membrane of renal mitochondrial cells and requires reducing equivalents. The renal mitochondria system is a three component system with mixed function monooxygenase and requires NADPH, molecular oxygen and magnesium ions The other components are renal ferredoxin reductase, an iron sulphur protein, and cytochrome p-450 (57).

Two forms of calcium are found circulating in the blood compartment: calcium bound to albumin (approximately 60%) and other circulating proteins which are in the free biologically active ionized calcium fraction (approximately 40%) which is needed for physiological functions (65).

Calcium is essential for the contraction of smooth, skeletal and cardiac muscles (66). In order to initiate smooth muscle contraction, calcium binds calmodulin thereby activating the myosin light chain kinase which allows for the interaction between actin and myosin proteins for muscle contraction (62,63). In cardiac muscle and skeletal muscle, calcium interacts with troponin C allowing a change in the conformation of troponin C and further interaction with actin and myosin causing muscle contraction (66). Calcium is not only important for muscle contraction but is the most important secondary chemical signaling messenger within cells (64). It promotes transmission of nerve impulses, excitation of nervous tissues, blood clotting and with the aid of sodium and potassium maintains transmembrane potential of cells (64,65).

The normal plasma levels of calcium are in the range of 2.12-2.65mmol/l (65). Approximately one gram of calcium must be provided in the diet every day and maintains a zero calcium balance (4).However, variability in calcium dietary intake is adjusted by the two key hormones PTH and $1,25(OH)_2D$ to maintain the optimal concentration of calcium (47). These hormones regulate calcium homeostasis in three tissues: kidney, intestine and bone (55,64,65) Changes in calcium concentration are monitored through the calcium-sensing receptors mainly in the parathyroid glands and renal epithelial tissue but also in the intestine, lung, brain, skin and other tissues (67).

## 1.3.3 Calcium Sensing Receptor (CaR)

Due to the vital importance of the calcium ion concentration and its critical role in many fundamental processes of the body, the distribution of the CaR has been found throughout the body (67). The CaR is present in the chief cells of the parathyroid glands, but also in the renal tubules including the proximal convoluted tubule, medullary and cortical thick ascending limbs, distal convoluted tubule and inner medullary collecting duct. Furthermore, it is located on the apical side of the inner medullary ducts which allow it to censor urinary calcium concentrations (68).

The CaR is a G-protein coupled receptor (GPCR) with a seven membrane-spanning segment and belongs to a subfamily of the large superfamily of GPCRs which also includes receptors for glutamate, GABA, pheromones, and odorants (Figure8)(69). It has a very large extra-cellular domain of approximately 600 amino acids which encompasses the $NH_2$ terminal domain which is heavily glycosylated and important for binding calcium ions. The 200 amino acids intracellular COOH-terminal tail includes 3 loops involved in coupling the receptor to its respective G proteins, thus linking CaR to intracellular signaling pathways .The biological active form of the receptor is a dimer linked by 2 disulfide bonds between the extra cellular domain (ECD) of 2 monomers. Binding of calcium to the ECD, modulates the functions of G proteins that couples it to intracellular signaling pathways. Initiation of signaling involves the binding of G-proteins to the receptor's intracellular loops ( loops 2 and 3) and the proximal portion of the C-tail (70).

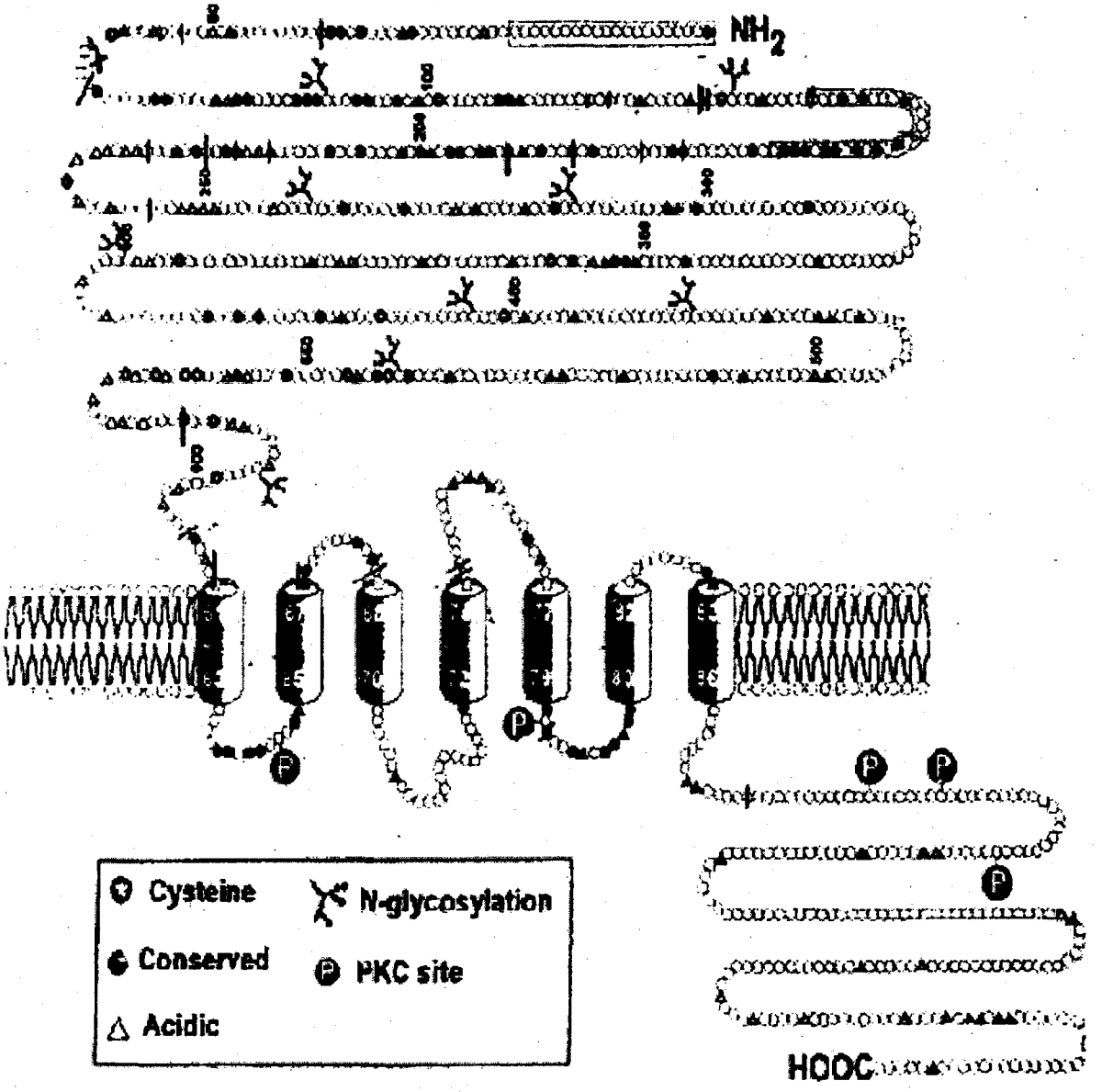



**Figure 8: Calcium Sensing Receptor (CaR) Schematic Representation.** The CaR is a G-protein coupled receptor (GPCR) with a seven membrane-spanning segment and belongs to a subfamily of the large superfamily of GPCRs. It has a large extra-cellular domain of approximately 600 amino acids which encompasses the $NH_2$ terminal domain which is heavily glycosylated and important for binding calcium ions. The 200 amino acids intracellular COOH-terminal tail includes 3 loops involved in coupling the receptor to its respective G proteins, thus linking CaR to intracellular signaling pathways (361).

Upon binding of calcium, CaR activates numerous intracellular signaling systems such as the phospholipases, Mitogen-Activated Protein Kinase (MAPK) and tyrosine kinases. PTH secretion is inhibited by a decrease in cAMP accumulation in parathyroid cells through modulation of the enzyme adenylate cyclase (71,72,73). An increase in extra cellular fluid concentration of calcium activates the CaR, phospholipase C and inhibits adenylate cyclase. As a result intracellular calcium increases via generation of inositol phosphates and cAMP decreases preventing exocytosis of PTH from secretory granules. Furthermore, CaR also exerts a suppressive action on parathyroid cellular proliferation and inhibits PTH gene expression (73). Additionally CaR regulates calcitonin secretion by the thyroidal C-Cells by stimulating PC-PLC and diacylglycerol for PKC-induced activation of sodium and calcium channels (74,75). This in turn allows entry of sodium and calcium into the cells producing depolarization which in turn activates the voltage gated calcium channels which further increases calcium entry and results in the secretion of calcitonin by C-cells(65,74,75,76). In summary, CaR regulates calcium homeostasis by several mechanisms namely through its effect on PTH and renal calcium resorption and possibly through calcitonin regulation.

## 1.3.4 Calcium Homeostasis: Integrated Mechanisms

PTH is produced by the chief cells located in the four parathyroid glands that are found behind the thyroid gland in the neck (77). PTH is an 84 amino acid single chain polypeptide synthesized as a larger protein, preproparathyroid hormone (preproPTH), which undergoes proteolytic cleavage to produce the PTH (78). This preproPTH undergoes proteolysis to form the proPTH which is then further cleaved to form PTH. PreproPTH has a 25 amino acid residue N-terminal signal sequence which is rich in hydrophobic amino acids and a 6 amino acid prosequence which is required for transport. Although PTH is produced as a full length molecule by parathyroid cells, the N-terminal

34 amino acids are sufficient for full activity and functionality of PTH (78). PTH acts via its G-protein linked receptor on the cell surface in the renal tubule cells and on osteoblasts (79,80).

PTH control of calcium homeostasis is depicted in Figure 9(80). PTH increases calcium absorption in the distal convoluted tubule (77,78,80). Furthermore, PTH causes phosphaturia via inhibition of phosphate uptake in the proximal convoluted tubules and also stimulates production of renal 1α hydroxylase leading to increased production of 1,25 hydroxyvitamin D which further mediates calcium absorption (81). PTH secretion by the parathyroid glands is regulated by a negative feedback loop that acts in response to concentrations of circulating calcium ions. As calcium ion concentrations increase, PTH synthesis and secretion is inhibited (82).

The key regulating step of vitamin D activation is 1 alpha hydroxylase in the kidneys which is stimulated by $1,25(OH)_2D$ itself in a negative feedback loop (56,61,62). Furthermore, $1,25(OH)_2D$ may to some extent increase calcium reabsorption in the proximal and distal convoluted tubules of the kidneys as well as phosphate reabsorption in the proximal convoluted tubules (53,54). $1,25(OH)_2D_3$ promotes intestinal calcium absorption at least in part through the synthesis of calcium binding proteins in the intestinal cells (55). Therefore, PTH indirectly controls calcium and phosphorus absorption in the intestine, specifically in the duodenum and jejunum through its regulation of $1,25(OH)_2D_3$ production.

The combined action of PTH and $1,25(OH)_2D$ to maintain serum ionized calcium concentrations in the 2.12-2.65mmol/L range is not achieved by their actions on the

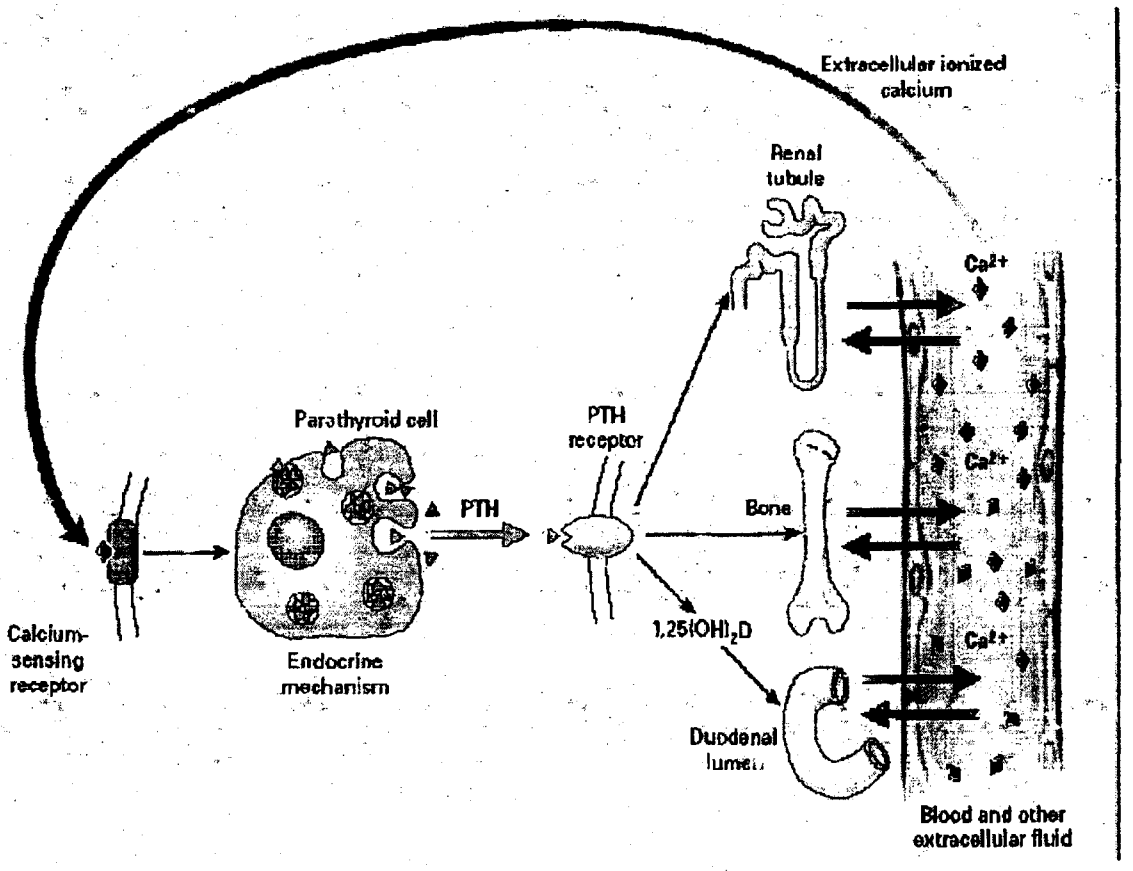



**Figure 9: Synthesis of PTH and its Effect on Calcium Homeostasis.** PTH is produced by the chief cells located in the four parathyroid glands that are found behind the thyroid gland in the neck It is a 84 amino acid single chain polypeptide synthesized as a larger protein which undergoes proteolytic cleavage to produce the PTH. PTH increases calcium absorption in the distal convoluted tubule and causes phosphaturia via inhibition of phosphate uptake in the proximal convoluted tubules. Morover, it also stimulates production of renal 1α hydroxylase leading to increased production of 1,25 hydroxyvitamin D which further mediates calcium absorption PTH secretion by the parathyroid glands is regulated by a negative feedback loop that acts in response to concentrations of circulating calcium ions. As calcium ion concentrations increase, PTH synthesis and secretion is inhibited **(47).**

kidneys and the intestine alone, but also through their action upon the skeleton (62). PTH and $1,25(OH)_2D$ are strong activators of osteoclastogenesis (86). PTH and $1,25(OH)_2D$ receptors are located on osteoblasts and upon binding of PTH and $1,25(OH)_2D$, RANKL is up regulated (19,20). RANKL is a type II transmembrane polypeptide protein essential for osteoclast differentiation via its receptor RANK located on the osteoclast membrane (87). RANK is a TNFR-related protein which is a type II transmembrane signaling receptor (21,87). The interaction between receptors RANKL and RANK results in the increased production and maturation of osteoclasts (87). This interaction is inhibited by osteoprotegerin (OPG) which is a soluble decoy receptor that inhibits osteoclast differentiation through its binding to RANKL (19). Upon RANKL activation, the osteoclasts release hydrochloric acid and collagenases to resorb bone, resulting in the mobilization of the calcium stores out of the skeleton in an attempt to normalize in circulating ion calcium concentrations (88).

## 1.3.5 Calcitonin

Calcitonin is a calcium-regulating hormone that regulates osteoclast activity when serum calcium concentration increases (89). Calcitonin inhibits bone resorption and promotes calcium deposition (90,91). However, calcitonin is not an essential calcium regulating hormone in humans and plays no role in the normal day to day regulation of plasma calcium concentrations. Clinical observations indicate that calcitonin deficiency in thyroidectomized or calcitonin excess in patients with medullary thyroid cancer have no visible effects on calcium homeostasis (92,93,94).

Calcitonin is secreted from the parafollicular cells (C cells) of the thyroid gland (95). It is a 32 amino acid polypeptide hormone formed from a larger precursor (procalcitonin). Pharmacologically, administration of calcitonin reduces plasma

concentrations of calcium and phosphorus (96). In contrast to PTH and $1,25(OH)_2D$ calcitonin receptors are formed on the osteoclast . The number and activity of osteoclasts decrease dramatically following binding of calcitonin to its highly expressed receptor on the osteoclasts and induces apoptosis (89). Furthermore, it blocks osteoclatogenesis induced by PTH (86,88,90). The biochemical mechanism of calcitonin is through its action on GPCRs and release of cAMP which bring upon the cellular effects (84,88,92). In addition, calcitonin acts on the kidneys to inhibit the resabsorption of calcium and phosphate (97).

## 1.3.6 "Classical" Physiological Action of Vitamin $D_3$

$1,25(OH)_2D$ is required for optimal health and is a vital component that exerts many regulatory effects on a vast number of tissues and organs (11). A major physiological function of vitamin D is the control of calcium homeostasis (12). Through the vitamin D receptor (VDR), it activates osteoblasts which transduce a signal to preosteoclasts which then differentiates into mature osteoclasts and then digests the matrix to resorb calcium from the bone. At the renal level, $1,25(OH)_2D$ acts on the distal convoluted tubule to increase calcium reabsorption and on the proximal tubule to increase phosphorus and calcium reabsorption (56). Furthermore, in the proximal tubule it acts as a negative feed back relay to inhibit the activity of 1αOHase and to stimulate the 24-hydroxylase activity (98). Furthermore, at the level of the small intestine and in the duodenum and jejunum, $1,25(OH)_2D$ actively stimulates absorption of calcium and phosphorus. It increases the synthesis of several of vitamin $D_3$'s binding proteins such as calbindin and increases the entry of calcium through the plasma membrane into the enterocyte, the movement of calcium through the cytoplasm and the transfer of calcium across the basolateral membrane into circulation (99). Calcium transportation in the

intestine is also mediated by the non-genomic effects of $1,25(OH)_2D$ through a mechanism known as transcalhatin (99).

## 1.3.7 "Non-Classical" Physiological Actions of Vitamin D

Early studies using autoradiograpy with labeled $1,25(OH)_2D_3$ indicated widespread tissue distribution of the VDR (107). Further studies later identified VDR in many of these tissues (100,107). This widespread distribution suggests that $1,25(OH)_2D_3$ may have additional roles than that of a mineral metabolism. It is now recognized that $1,25(OH)_2D_3$ plays a central role in the immune system, cell growth, differentiation and cancer (113,114,311). Tissue expression of the VDR include parathyroid glands, the islet cells of the pancreas, bone marrow cells and the keratinocytes of the skin (4,11). VDR has also been identified in breast, colon, prostate, heart, brain, skeletal muscle, monocytes and activated T and B cells as well as numerous cancer cell lines and tissues (100,101).

The localization of the VDR in the parathyroid gland suggests an interplay between PTH and $1,25(OH)_2D$ (78). It has been further demonstrated that the two hormones worked together in a regulatory feedback network (102,111,112). Furthermore it has been shown that $1,25(OH)_2D$ controls parathyroid cell growth and suppresses synthesis and secretion of PTH (103). $1,25(OH)_2D$ was first shown to regulate growth and differentiation of bone marrow cells (104). Identification of VDRs in promyeloctic leukemia cells (HL-60) lead to studies showing $1,25(OH)_2D$ causes growth inhibition and induces differentiation into monocyte-macrophages (105). Subsequently, similar results were found in mouse myeloid leukemia cells (m1), breast, colon, prostate, skin and lung cell lines (106,107,108,109). In keratinocytes, addition of $1,25(OH)_2D$ causes skin cells to differentiate and suppresses proliferation. As a result $1,25(OH)_2D_3$ analogues have

been devised in the treatment of the hyperproliferative skin disorders such as psoriasis (110).

Vitamin D has been shown to be a modulator of the immune system (113). The VDR was identified in mature CD-8 T lymphocytes (104,105,114).Further studies indicated that 1,25-dihydroxyvitamin $D_3$ is beneficial in experimental autoimmune encephalomyelitis, rheumatoid arthritis, systemic lupus erythematosus, type I diabetes, and inflammatory bowel disease (113,115).

In the pancreas vitamin D deficiency enhances glucose-mediated insulin secretion through VDR mediated modulation of calbindin expression and control of intracellular calcium flux in the islet cells (116,117). In addition, new roles for vitamin D are under investigation in particular in reproduction and in the nervous system (118). Thus, vitamin D is now being recognized not only as a central regulator of calcium and phosphorus homeostasis but also as a regulator of numerous target organs in health and disease (Figure10).

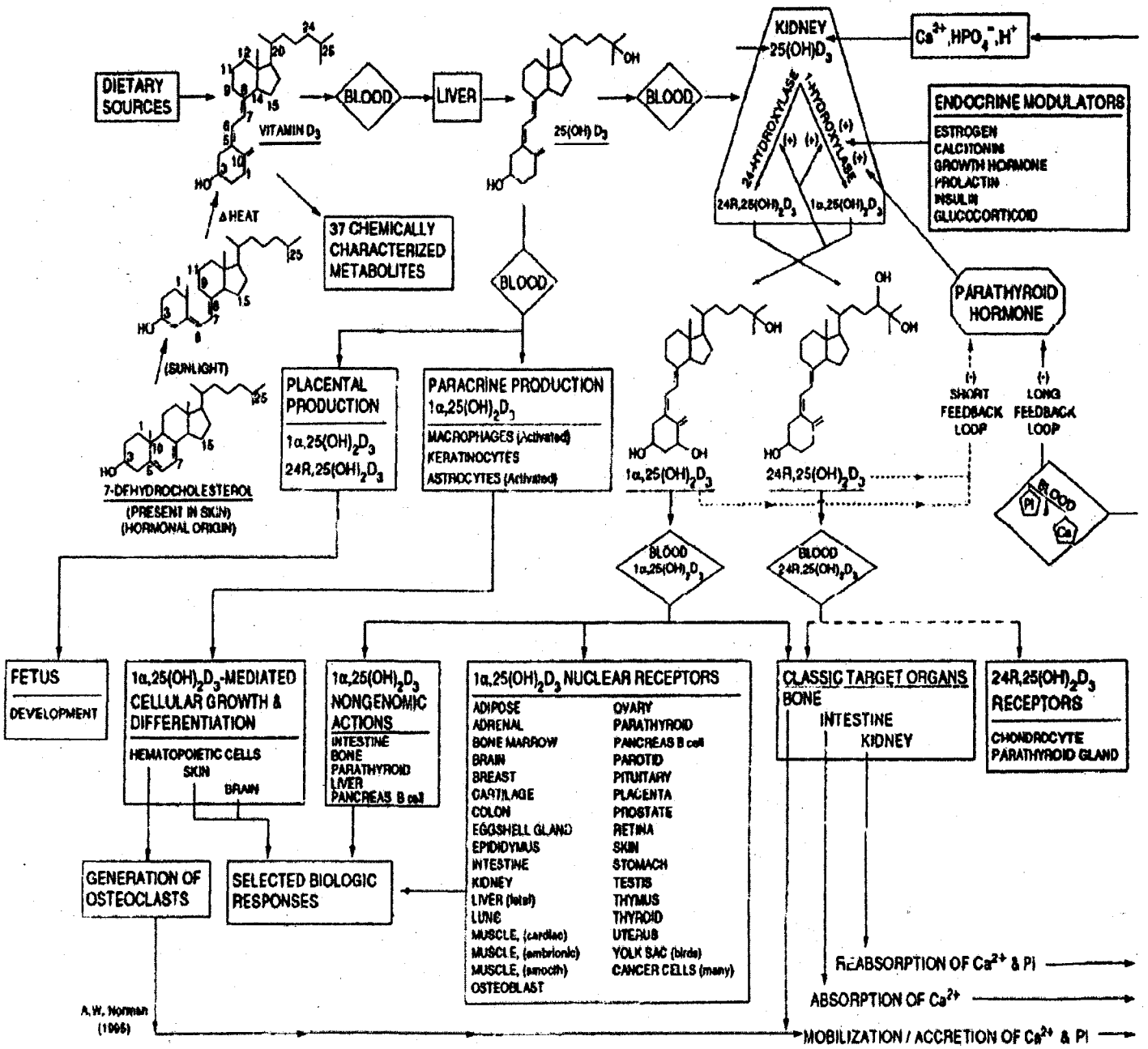



**Figure10: Integrated Effects of 1,25 dihydroxyvitamin D on the Human System.** 1,25dihydroxyvitamn $D_3$ is a pluripotent seco-steroid with pleiotropic actions that have effects on a diverse number of biological systems. The above figure summarizes vitamin $D_3$ synthesis process; it affects in conjunction with the PTH on calcium homeostasis and it physiological effects/non physiological effects that range from immunological stimulation to roles in differentiation, growth and even reproduction. **(132).**

## 1.4 Nuclear Receptors: Ligand-Responsive Transcription Factors

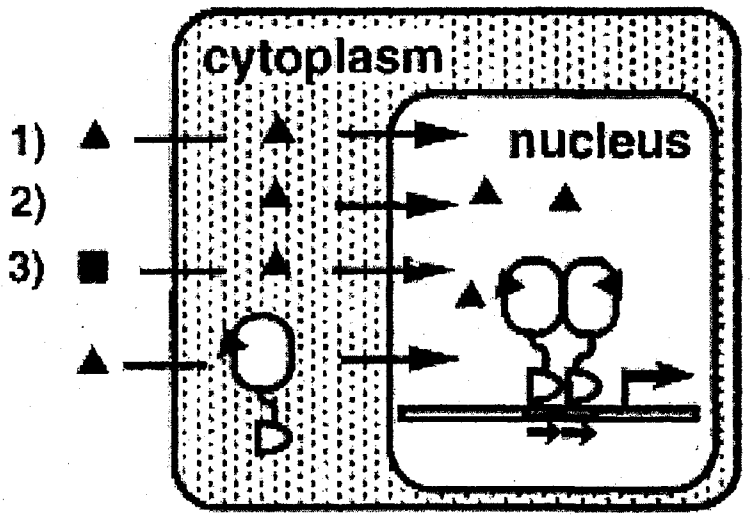
A variety of lipid soluble hormones are secreted by endocrine cells including corticosteroids that control metabolic states, sex steroids that regulate reproductive processes and thyroid and retinoid hormones that control developmental processes (119,120,121,153). These hormones act through the activation of a family of structurally related ligand-activated transcription factors called nuclear receptors (119,120). The VDR is a classical example of a nuclear receptor which when bound to its ligand triggers intracellular signals (120). $1,25(OH)_2D$ and most lipid soluble hormones have intracellular receptors which constitute the nuclear-receptor super family. These nuclear receptors have several domains including a N-terminal region (A/B region) of variable length of 100-500 amino acids, a DNA binding domain characterized by a repeat sequence of the $C_4$ zinc-finger motif and a C-terminal domain containing the hormone binding domain. Important features include 1) The DNA binding domain with its two zinc fingers 2) The hormone binding domain with its 12 α-helices and 3) The two activation domains AF1 and AF2 which can also function as repressor domains. AF1 contributes to ligand independent action of the receptor whereas AF-2 is strictly ligand dependent and highly conserved (119,120,121,122).

Nuclear receptors have been classified in two variants (Type 1 and 2) based upon their half-site sequences and organization (123). Type 1 receptors comprise steroid hormone receptors such as the glucocoticoid, estrogen and androgen receptors which bind to DNA sequences as homodimers to inverted repeat with a 3 base pair spacer. Glucocorticoid, androgen, progesterone and mineralocorticoid receptors all bind the same

half-site AGAACA and their specificity lies in bases adjacent to this half site (120,124). Type 2 receptors such as the VDR, retinoic and thyroid hormone receptor bind non-steroid hormones and interact with DNA sequences on direct repeats of AGGTCA. Specificity depends on the spacing between the repeats. Type1 and Type 2 receptors also differ in their location and mode of activation. Type 1 receptors in resting cells are held in the cytoplasm complexed with a variety of chaperone proteins including hsp90(Figure11)(120,121,122). Following hormone binding to its receptor binding domain, the receptor is released from hsp90 complex and translocates into the nucleus (124). In the nucleus, the receptor-hormone complex binds the appropriate hormone response element (HRE) and activates or represses gene expression (124,125). Type 2 receptors are always found in the nucleus loosely bound and the HRE in the absence of hormone (121,124). Addition of the hormone causes a conformational change in the receptor and recruitment of transcriptional cofactors (120,121,123).

Nuclear receptors bind HRE as dimers, either homo or heterodimers (125).HRE are made up of two half-sites separated by a spacer of between 1-5 nucleotides. Receptors that bind such sequences do so as heterodimers with another receptor (120,124,125).

A

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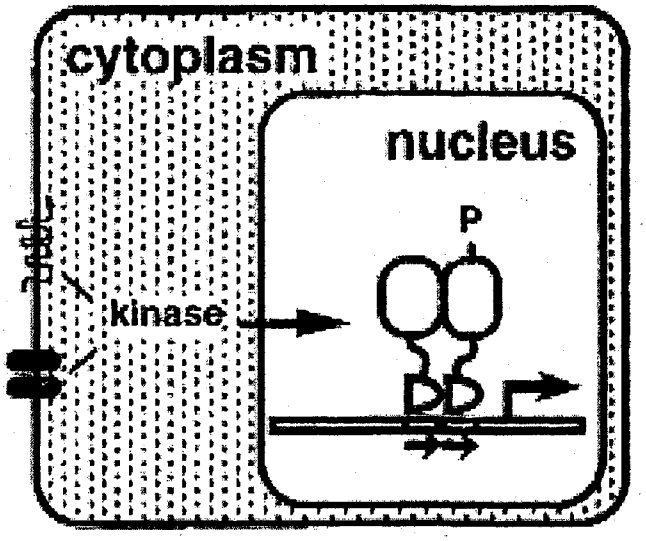



**Figure 11: Schematic Representation of Type 1 and Type 2 Nuclear Receptors. (A)** Type1 Nuclear receptor resides in the cytoplasm and upon activation translocates into the nucleus where they bind to their response elements. **(B)** Type 2 Nuclear Receptor resides in the nucleus and upon activation change conformation so that they can bind the necessary response elements (137).

## 1.4.1 The Molecular Mechanism of Action of Vitamin D

The first step of 1,25dihydroxyvitamn D action begins with its binding to the VDR in target organs (126). The VDR hormone complex is tightly associated with the nucleus which then modulates transcription of DNA into messenger RNA and subsequently translation of proteins that carry out its biological actions. The VDR system shares many common features with the retinoic acid receptor (RAR) and the thyroid hormone receptor (TR) including heterodimerization with the retinoid X receptor (RXR), binding as a heterodimer to HREs and recruitment of coactivators and corepressors (Figure12)(127,128,129).

Upon binding of $1,25(OH)_2D$ to cytoplasmic VDR, the complex rapidly translocates into the nucleus along microtubules (130). This rapid nuclear localization is mediated by two potent nuclear localization signals which have been identified in the VDR. The first is a bipartite signal consisting of a cluster of basic residues and the second is a basic sequence of seven amino acids residues (49-55) which are unique to the VDR (131). After translocation into the nucleus, the VDR heterodimerizes with the RXR. This dimerization occurs at a specific interface on the VDR (128). Heterodimerization of the ligand-activated VDR with RXR induces a conformational change of the VDR that is necessary for VDR transactivating function through its response elements in the promoter regions of target genes (125,132). Several VDREs have been characterized in the promoter region of vitamin D regulated genes (133).

VDREs directs the VDR-RXR heterodimer to the promoter region of $1,25(OH)_2D$ regulated genes with the RXR binding the 5' half site and the VDR occupying the 3' half site (132,133). Binding of the heterodimer to the response element induces a bend in the DNA of the promoter (133). Furthermore, the binding of $1,25(OH)_2D$ to the VDR causes a change in conformation of the receptor that permits the AF-2 domain of the VDR to interact with other transcription factors, coactivators and corepressors (132,133). The transactivation domain of the VDR serves as an adaptor surface for nuclear proteins that are essential for the VDR-mediated transcriptional regulation (134). Upon this binding recruitment of numerous coactivators and corepressors occurs; such as TFIIB, SRC-1, NCoA-62,TATA binding protein (TBP), associated factor TAFII28, TIF1 and CBP/p300 (133,135,136,137,138). SRC-1 and CBP/p300 possess histone acetlytransferase activity, which modify nucleosome structure and exposes the DNA for transcription (138). Thus, the net result of the binding of the ligand receptor is the facilitation of the preinitiation complex and the regulation of transcription of target genes by RNA Polymerase II leading to the translation of proteins (Figure 12)(137).

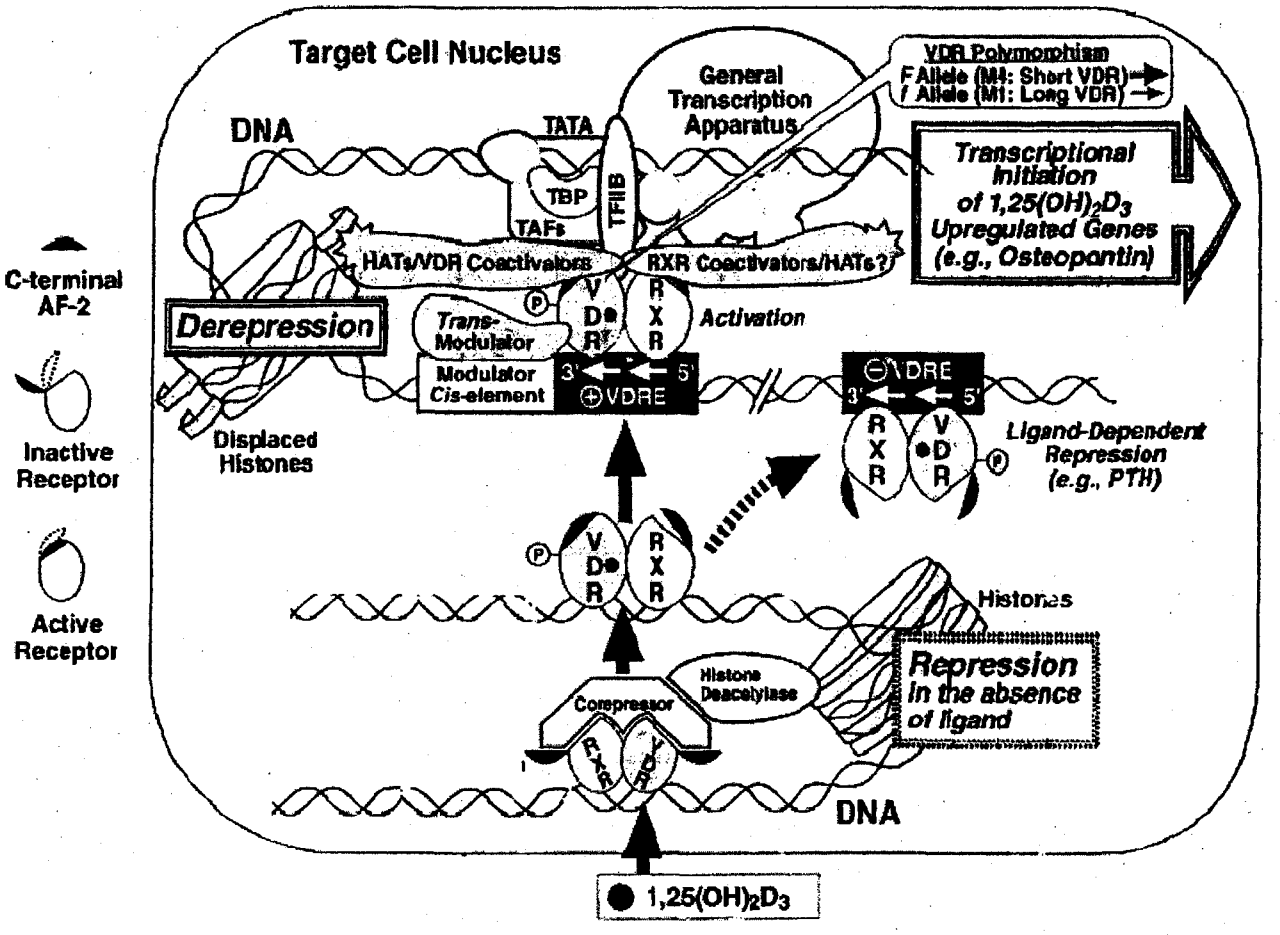



**Figure 12: Molecular Mechanism of Action of 1,25 dihydroxyvitamin D.**Upon binding of $1,25(OH)_2D$ to cytoplasmic VDR, the complex translocates into the nucleus along microtubules where the VDR heterodimerizes with the RXR. Heterodimerization of the ligand-activated VDR with RXR induces a conformational change of the VDR that is necessary for VDR transactivating function through its response elements in the promoter regions of target genesThe binding of $1,25(OH)_2D$ to the VDR causes a change in conformation of the receptor that permits the AF-2 domain of the VDR to interact with other transcription factors, coactivators and corepressors .The net result of the binding of the ligand receptor is the facilitation of the preinitiation complex and the regulation of transcription of target genes by RNA Polymerase II leading to the translation of proteins (133).

## 1.4.2 Vitamin D Binding Protein

Previtamin D is transported from the skin into the circulation by entering with chylomicrons in the small intestine through the lacteal system (49,51,53). Vitamin $D_3$ then circulates bound to its own transport protein termed the vitamin D binding protein (DBP). DBP is a multifunctional, highly expressed, polymorphic serum protein (139). It is a member of the albumin (ALB) α fetoprotein (AFP) and ά albumin (ALB)/afamin (AFM) gene family (139,140). It is encoded on human chromosome 4, specific to the region to bands 4q11-q13 (140). Its main site of expression and synthesis is in the liver which produces a 458 amino acid (AA) polymorphic human serum protein whose exact size idepends on its glycosylation (139,141).Upon secretion; this 458 amino acid protein undergoes cleavage of its 16-amino acid signal peptide. It has strong homology with ALB, AFP and ALB/AFM (141). The DBP is characterized by 28 Cys residues in the disulfide form and a triple domain modular structure. DBP has two binding regions: a vitamin D binding domain between residues 35 and 49 and an actin binding domain between residues 373 and 403 (142).

The major function of the DBP is binding vitamin D and transport of vitamin D to its target sites (Figure 4). DBP also binds 25OHD (139). DBP circulates at a concentration 20 fold higher than 25OHD in order to limit and control its delivery (139,143).When complexed with $25OHD_3$, DBP is reabsorbed in the proximal renal tubules preventing its urinary loss and targeting the metabolite to the tubular kidney cells site of the 1 alpha hydroxylation (144). Additionally, it increases the half life of circulating vitamin D metabolites and protects from dietary vitamin D deficiency as demonstrated from experiments with DBP knock out mice (145).

DBP has other functions than vitamin D transport as indicated by its binding domain to monomeric actin preventing its polymerization into filamentous actin (146). Additionally, it works in concert with serum protein gelsolin (GSN) to protect the microcirculation from actin mediated damage (146,147). Recent evidence also supports a role for DBP in the immune system by activating macrophages (147).

### 1.4.3 Vitamin D receptor (VDR)

VDR, a member of the nuclear hormone receptor superfamily, has been localized to chromosome 12 (119). The LBD has high affinity for $1,25(OH)_2D$ with a $Kd=10^{-10}$ to $10^{-11}$ M (126). The truncated $NH_2$–termed A/B domain of 20 amino, is followed by the DNA binding domain (DBD) up to 90 amino acids. A hinge region links the DBD and LBD. The DBD is characterized by the presence of two zinc finger DNA binding motifs between residues 24 and 90 (129). The two nuclear localization signals are located on a stretch of seven amino acids in the zinc fingers DNA binding motifs and a bipartite signal made of a group of basic residues (129,131). The C–terminal region extends from residue 227-422 and comprises the LBD. Furthermore, it interacts with the RXR upon activation by $1,25(OH)_2D$ (Figure 12).The RXR dimerization surface with the VDR occurs in the in the first zinc finger and in a heptad repeat in the LBD (148). The selective association between the VDR and RXR is determined by a Asn residue, in the first zinc finger, Lys and Glu in the T-box COOH-terminus of the second zinc finger and two of the heptad repeats in the LBD as determined by the crystal structure of the VDR (132). The C-terminal binding domain termed the AF-2 domain is critical for transcription and if removed results in decreased ligand binding affinity and loss of transcriptional activation (134). The 1 alpha and 25-hydroxyl groups are crucial for high affinity binding and their

absence results in a 500 fold decrease in affinity of $1,25(OH)_2D$ for its receptor (133). Alteration to the amino acids C-terminus results in decreased affinity whereas deletion causes complete loss of ligand binding (132,133,134).The DNA binding domain contains 9 cysteine residues that are strictly conserved, the first 8 cysteine residues are responsible for high affinity interaction with specific DNA sequence in the promoter region of target genes (134).

The VDR once activated by its ligand heterodimerizes with RXR and binds to VDREs. The VDR then undergoes conformational changes creating a "docking site" or an adaptor surface for the recruitment of coactivators and corepressors (133,134). It is the transactivation domain of the VDR that serves as this adaptor surface. Once this conformation has taken place, the AF-2 domain then interacts with components of the transcriptional initiation complex and nuclear transcriptional coactivators (137).

## 1.4.4 Retinoic X Receptor (RXR)

RXR like other nuclear receptors contains a $NH_2$ (A/B) terminal domain; a C domain which binds DNA and a COOH-terminal (D/E) domain which binds its ligand and activates transcription. 9-cis retinoic acid has been identified as an RXR ligand (148). Upon ligand binding RXR, conformational changes allowing for interactions and recruitment of coactivators and corepressors occur (133,134). Heterodimerization of the RXR occurs through two interfaces; one in the DNA binding domain and the other in the ligand–binding domain (150). Ser 260 located in the omega loop between helices one and three are extremely important for VDR/RXR heterodimerization and transcriptional activation (133,134). It has been identified as a phosphorlation site by MAP/ERK pathway that when phosphorylated causes conformational changes in the RXR and disrupts interactions with coactivators leading to vitamin D resistance (151).

## 1.4.5 Vitamin D Response Elements (VDRE)

Hormone Response elements (HRE) are short DNA sequences that influence transcription (124). They are usually located in regulatory sequences or in near proximity to the promoters or even found in enhancer regions several kilobases away from transcriptional initiation site (153). Two consensus motifs AGAACA and AGGTCA are recognized by steroid class III receptors while the rest of the receptor's superfamily recognizes the second sequence. Some receptors bind a single hexameric motif but most receptors like the VDR bind as a homo or heterodimer to the response elements (154). Response elements consist of two core hexameric motifs or dimeric with half sites arranged as palindromes, inverted palindromes or direct repeats as in the case of VDRE (134). VDREs consist of two repeat AGGTCA sequences separated by several non specific bases (Figure12) (154). This variable number of nucleotides determines the receptor specificity; VDRs half site is separated whereas RXR is separated by a single nucleotide (154,155).

Upon binding of the VDR/RXR complex, VDREs in the response elements bend in the order of 30°degrees (156). This bend is followed by the recruitment of coactivators and corepressors which contain acetyl and deacetyls activity promoting transcription by making the promoter more accessible to transcriptional factors (137).

## 1.5 Transcription Factors:

Conformational changes in the VDR and bending of VDRE's activates the pre-initiation complex. In order to initiate transcription, promoters are recognized by transcription factors, including the basal transcription factors that interact with the core promoter elements as well as sequence specific transcription factors. The VDR interacts

directly with a number of transcriptional factors and with coactivators/corepressors (Figure 12).

## 1.5.1 TFIIB

Upon binding of the TATA box binding protein (TBP) to the TATA box, TFIIB is recruited and allows for the recruitment of other transcription factors that position RNA polymerase II over the TATA box and initiates transcription (Figure12)(157). TFIIB is a 30kDA protein and interacts with the VDR region of approximately 70 amino acids in the resting state. TFIIB is sequestered in the VDR complex and upon ligand binding, TFIIB is freed and catalyses the preinitiation complexes recruitment (161).

## 1.5.2 Co activators and Repressors:

Coactivators and corepressors regulate the assembled preinitiation complex by either enhancing or repressing transcription (Figure12) (135). Coactivators are linking proteins that interact with the basal transcriptional machinery, while corepressors bind to coactivators and inhibit the formation of activation complexes (137). These interactions are mediated through the COOH terminus of the LBD (148,161). Recruitment of coactivators and corepressors such as SRC/p160, NCoA-62/ski, CBP/p300, nuclear corepressor (NCoR), silencing mediator for retinoic and thyroid hormone (SMRT) and RIP 140 to the VDR- RXR complex then follow in an orderly manner (160-179).

## 1.5.3 SRC/p160 Co-activators

The steroid receptor coactivators (SRC) family of coactivators include SRC-1, SRC-2 (GRIP,TIF2) and SRC-3 (ACTR,TRAM,AIB1)(162,163,164).Unique to this family of coactivators is the sequence comprised of the series of LxxLL motifs with conserved spacing between the motifs (165). It is this sequence that directly contacts the AF-2 domain of nuclear receptors and also interacts with the VDR in a ligand dependent

manner. Characteristic of this family of proteins is the histone acetyl transferase (HAT) activity (166). These coactivators act as bridging proteins that link the receptor to RNA polymerase II and the basal transcriptional machinery which recruits the components of the preinitiation complex of transcription (167). SRC-1 and SRC-2 interacts with TFIIB, TATA binding protein and CBP/p300 through their distinct C-terminal domain as does SRC-3 with the CBP/p300 (167,168).

## 1.5.4 NCoA-62/ski-Interacting Protein

NCoA-62/ski is a protein that interacts with the v-ski oncoprotein and therefore was called ski-Interacting Protein (SHIP) (169). Ski is a cellular differentiating factor that functions through nuclear receptor signaling pathways (164). Similar to SRC it interacts directly with the VDR. However it does not contain LxxLL motifs and lacks HAT activity, but acts by forming ternary complexes with VDR and SRC coactivators (165,169).

## 1.5.5 CBP/p300

CBP/p300 is a multi-domain protein which weights approximately 400Kda (Figure 12). One domain of CBP/p300 binds the phosphorylated acidic activation domain of CREB transcription factors, while the other has histone acetylase activity (170). Structurally similar to all proteins collectively termed the p160 coactivators that include complexes such as the SRC/p160 coactivators, it contains the same structural motif LxxLL as well as the AF-1(A/B), DBD(C),CoR(D), AF-2- LBD(E) and a F region (171). This bridging protein together with HAT undergoes a conformational change which upon ligand binding of the alpha helix which contains the AF-2 core helix in the LBD domain forms a charged clamp that accommodates other coactivators and corepressors within the hydrophobic cleft of the LBD (171,172).

This complex has diverse functions but is primarily associated with acetylase complex on nucleosomes, where it acetylates histone tails thus facilitating the interaction of general transcription factors with the promoter DNA (172). Its interaction with TFIID, TFIIB and RNA polymerase holoenzyme are associated with its function. CBP/p300 also participates in various tumor suppressor pathways and has been shown to interact with the tumor suppressor p53 (173).

## 1.5.6 RIP140

The nuclear receptor superfamily comprises ligand regulated transcription factors that share common modular structure and regulate transcription. Receptor interacting protein of a molecular weight of 140 Kda (RIP 140) was isolated through its recruitment by estrogen receptor alpha (ERα) AF2 in the presence of ligand and was subsequently shown to interact with many nuclear receptors such as α/β RAR, α/β RXR and α/β TR (174,175). It is located on the human chromosome 21 in the q11,2 region (176).

RIP140 is a corepressor comprised of 1158 amino acids and contains two nuclear localization signals (NLS) located at position 97 and 856. It contains nine LxxLL motifs spread throughout the molecule giving the specificity that is required for interactions with different receptors (178). RIP140 contains a great amount of sequence homology with the SRC and CBP/p300 family (172,178). Thus, its transcriptional repressor activity is mediated by competition for binding sites due to the shared sequence homology and its intrinsic histone deacetylases transferase activity (HDAC) (174-179).

## 1.6 Non-Genomic Effects of Vitamin D

1,25-dihydroxyvitamn D is a secosteroid which acts primarily by modulating transcription but also has been shown to activate non genomic pathways that regulate calcium transport (17). There is now evidence for a membrane bound VDR that elicits non genomic response through second messenger activation such as MAP Kinase or cyclic AMP and ultimately influencing the activity of calcium channels (180). These non-genomic effects are dependent upon concentration levels of the hormone as well the extent of receptor binding (Figure 13) (181). Other non-genomic, non calcemic effects have also been described such as the alterations of the physicochemical properties of membranes like fluidity, phosphlipid turnover and the microenvironment of proteins (182).

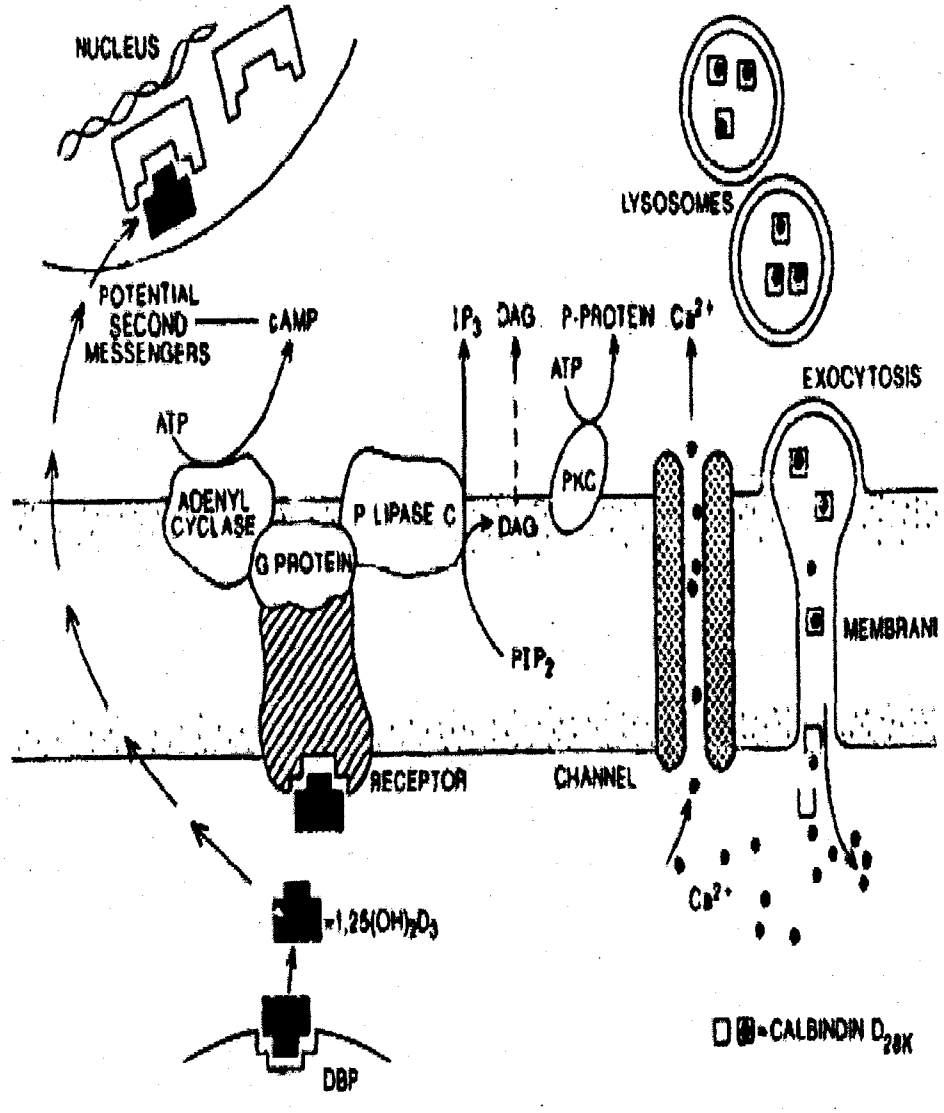



**Figure 13: Non-Genomic Regulation of Calcium Via 1,25 dihydroxyvitamin $D_3$.** This is mediated through signal transduction of the opening of voltage gated Ca 2+ channels by 1,25 dihydroxyvitamin $D_3$ . 1,25 dihydroxyvitamin $D_3$ interacts with secondary messaging systems such as the MAP Kinase or cyclic AMP which influence the activity of calcium channels through a variety of membrane bound signaling proteins which open up the voltage gated Ca 2+ channels (181).

## 1.7 1,25-dihydroxyvitaminD Interaction with Growth Factor/Cytokine Synthesis and Signaling Pathways

1,25-dihydroxyvitamin D is a hormone that acts on diverse cell types to regulate development, growth and differentiation (183). Examples of this are cartilage, haematopoietic cells, bone cells, mesenchymal, neural vascular endothelial, immune cells and keratinocytes (184). These effects are mediated at least in part by modulating gene expression of receptors such as G-protein coupled receptor, TGF Beta receptor, cytokine receptor and receptor tyrosine kinase (185,188). $1,25(OH)_2D$ also regulates the expression of growth factor/cytokine synthesis thereby modulating the process of growth and differentiation (186). In keratinocytes 1,25-dihydroxyvitamn D induces keratinocyte differentiation and suppresses keratinocyte growth by interacting with a multitude of growth factor/cytokine signaling pathways (187). These include mitogen-activated protein kinase (MAPK), transforming growth factor beta (TGF β–BMPs), cytokine signaling (JAK-STAT pathway), Nuclear factor kappa β signaling (NF-kB) and secondary signaling path ways such as phosphatidylinositol-3phosphate kinase (PI3K), inositol 1,4,5 trisphosphate and diacylglycerol pathway (IP3/DAG) to mediate these effects (Figure14)(189,190). The different signaling pathways have overlapping signaling components that can activate one or a combination of signaling cascades. Thus, the action of $1,25(OH)_2D$ involves multiple signaling mechanisms adding to the complexity and the lack of understanding of vitamin D signaling.

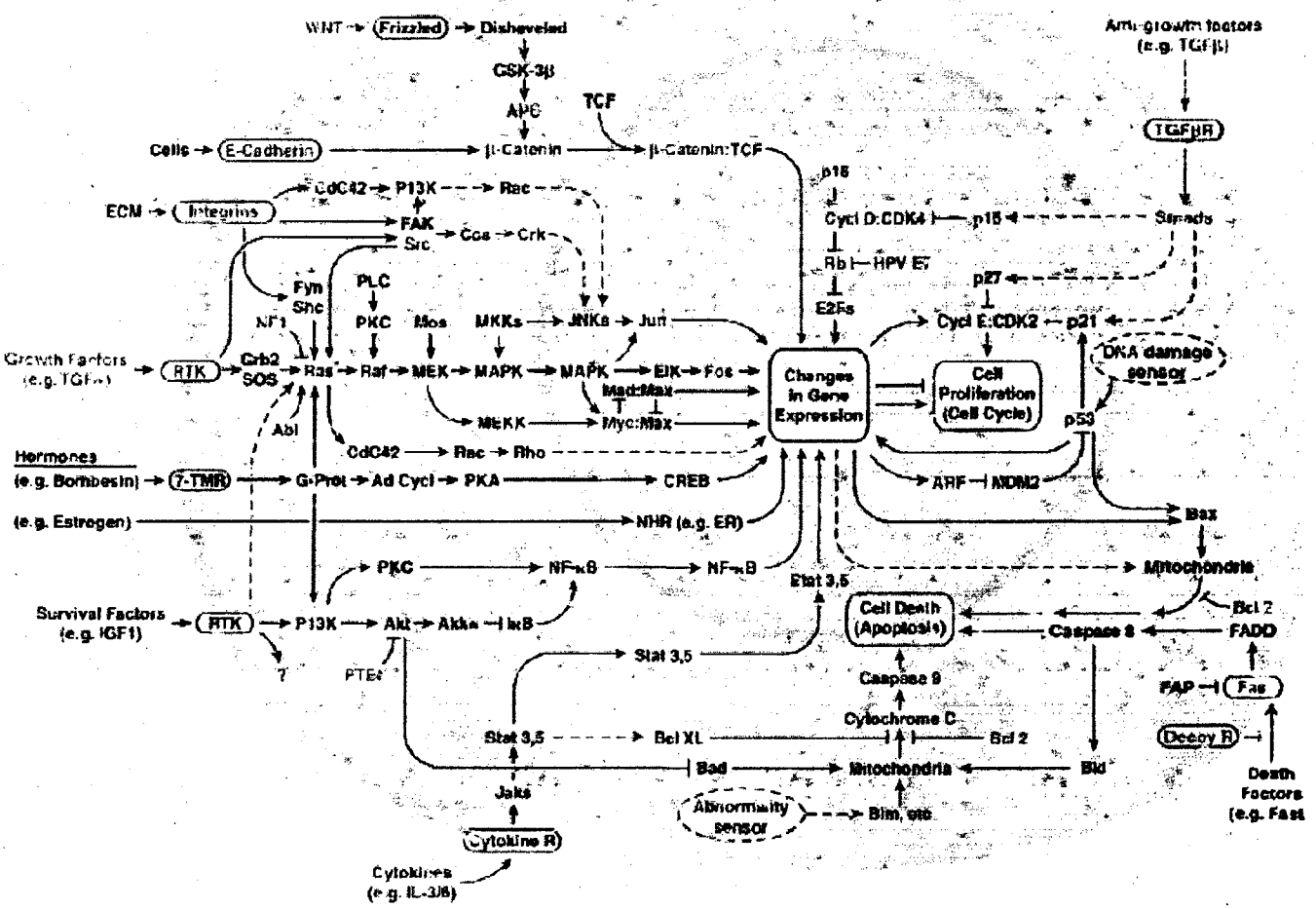



**Figure 14: The Multiple Signaling Pathways Involved in Cell Signaling** (248).

## 1.7.1 Mitogen-Activated Protein Kinase Signaling Pathway (MAPK) and Vitamin D

The MAP kinase cascade is activated by receptor tyrosine kinases (RTK) which have intrinsic protein tyrosine kinase activity. Examples of these receptors are fibroblast growth factor receptor, epidermal growth factor receptor and platelet-derived growth factor receptor (191,192,193). The receptor tyrosine kinase protein families are encoded by a large number of genes: 58 in the human genome. These are divided into 19 subfamilies which differ mainly in the structures of their extra cellular domains due to the interactions with different ligands (194,195). RTK are characterized by an extracellular domain containing a ligand binding site, a single transmembrane alpha helix and a cytosolic domain that contains the tyrosine kinase activity (196). The intracellular kinase domains are highly similar in all types of RTK. 1,25-dihydroxyvitamin D has dramatic effects on RTK ligands by stimulating signaling through the rapid response steroid binding protein and through activation of membrane associated tyrosine kinases which activate the RAS and a multiple of secondary pathways (353,354). Moreover, PDGF and NEGF synthesis are up regulated by $1,25(OH)_2D$ causing inhibition of cell growth (197,198,353).

Signaling involves hormone induced activation of the tyrosine kinase enzyme, which then initiates a response through a variety of pathways (Figure 14, 15). RTK ligand binding to extracellular domains causes the formation of receptor dimers which then allows for the kinase to phosphorylated tyrosine residues in the activation loop of other subunits (199). These phosphorylated tyrosine residues then serve as docking sites for GRB2 which is a linking/adaptor protein that contains SH2 domains that bind phosphorylated tyrosine residues.GRB2 contains two SH3 domain that bind to a proline

rich sequence and activate SOS (son of sevenless) which is a guanine nucleotide exchange protein which activates RAS (195,200). RAS is a monomeric GTP binding protein that requires SOS to catalyze conversion of inactive GDP bound RAS to active GTP bound RAS (201). Activated RAS then promotes the formation of membrane signaling complexes containing three sequential acting protein kinases. Active RAS binds the N-terminal regulatory domain of RAF which is a serine/threonine kinase. In the resting state, the RAF N-terminal regulatory domain binds to the catalytic domain and inhibits its activity (200,201,207,269). Activated RAS GTP binds the RAF N-terminal regulatory domain causing removal of phosphate from the N-terminal regulatory domain, release of 14-3-3 and addition of phosphates to the catalytic domain that activates the Raf kinase enzyme (202). Active RAF then phosphorylates and activates MEK kinase which in turn then phosphorylates and activates MAP (serine/threonine). MAP is a dual specificity kinase that adds phosphates to both serine/threonine and tyrosine residues on the MAPK enzymes. Addition of these two phosphates causes activation of MAPK, and movement into the nucleus to act on various transcription factors (203). Activated MAPK forms a dimer that phosphorylates another kinase, p90RSK, in the cytoplasm. Activated MAPK dimer is transported to the nucleus where it adds two phosphates to ternary complex factor (TCF). Activated p90RSK moves to the nucleus where it adds a phosphate to serum response factor (SRF). Two SRFs and one TCF then form a trimeric complex with binds to the SRE of the c-fos enhancer (and several other enhancers) to activate gene expression. C-fos is a transcription factor whose expression is up regulated by growth factors (204).The MAP kinase pathway can be activated through different

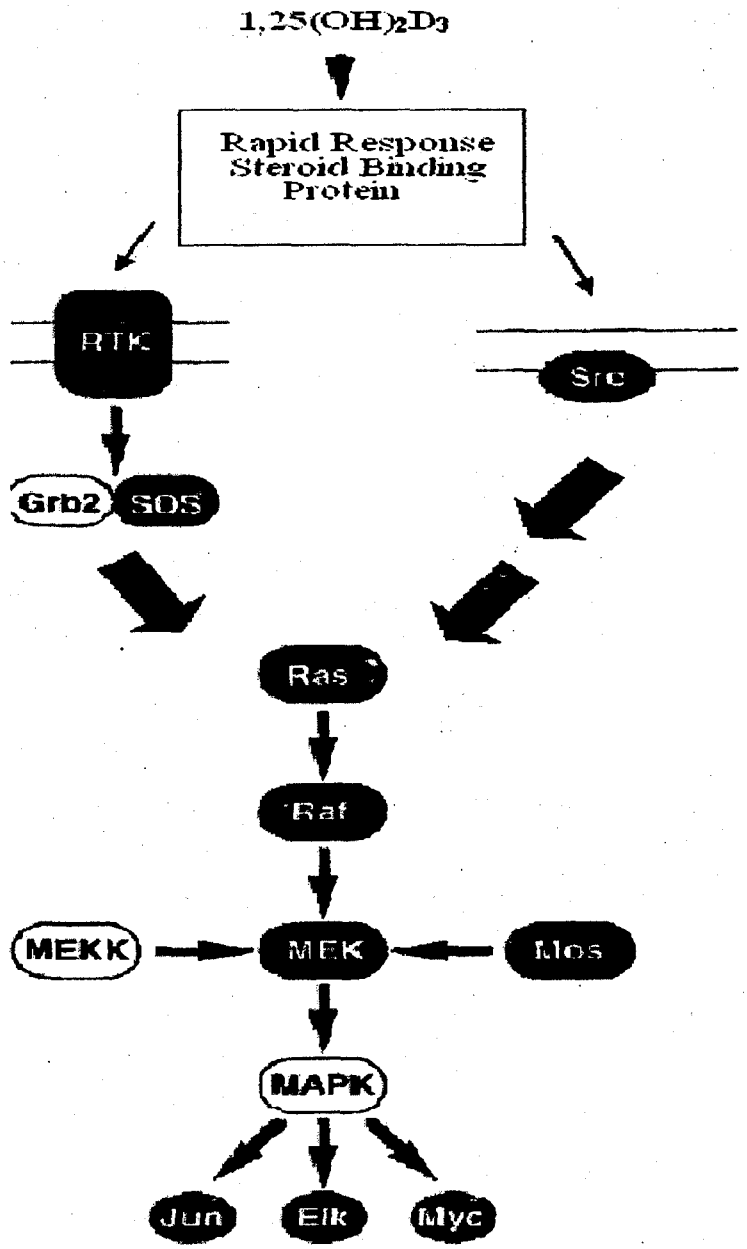



**Figure 15: RAS Signaling Transduction Pathways with 1,25 dihydroxyvitamin $D_3$.** 1,25-dihydroxyvitamin D effects on RTK ligands are mediated through the rapid response steroid binding protein (RRSBP) and through activation of membrane associated tyrosine kinases which activate the RAS and a multiple of secondary pathways. Upon activation of a series of cellular intermediates, activation of the results in the movement into the nucleus where it act on various transcription factors (257).

receptors other than RTK such as oncogene Mos which activate MEK and other activating stimuli such as ionizing radiation, hydrogen peroxide and UV light (Figure 14,15)(205,206,207).

Several studies have documented the relative resistance to 1,25-dihydroxyvitamin $D_3$ at high concentrations. It has been documented that in a keratinocyte model of tumor progression (HPK1A /HPK1ARAS) at high concentrations of 1,25-dihydroxyvitamn $D_3$ the growth inhibitory effects are diminished. These effects are due to the RAS-MAPK pathway which phosphorylates the RXR at SER260 causing the RXR to undergo a conformational change which causes the decrease in responsiveness to $1,25(OH)_2D_3$ (151,339).Thus, modulation of the RAS-MAPK pathway is extremely important for $1,25(OH)_2D_3$ functionality and efficacy.

## 1.7.2 Transforming Growth Factor Beta (TGF-ß)Signaling Pathway and Vitamin D

The TGF-ß families of regulatory factors, also called morphogens, are soluble factors secreted by one cell to regulate gene expression in other cells (208). There are a total of about 30 different genes for these factors in humans which are divided into two major subgoupings: 1: the TGF-ß subgroup that includes the TGF-ßs (transforming growth factor ß) and the activins 2: the BMP/GDF subgroup (bone morphogenetic protein, growth and differentiation factor (209)

Members of the TGF-ß family are important for controlling critical steps in development and are expressed at precise developmental stages by specific cells in the embryo to control cell fate (208). TGF-ßs act to inhibit uncontrolled proliferation of mammalian cells (210). Many human cancers are characterized by mutations in TGF-ßs,

their receptors and signal transducing factors (211). In keratinocytes,1,25 dihydroxy-vitamn $D_3$ causes the activation of TGFβ signaling cascade that results in the increase production of TGF β which is a major growth-inhibitory autocrine factor (212).

TGFβ proteins share homologue structure and are secreted as a large precursor proteins including a pro-domain. After secretion, the precursor is cleaved by a protease, but the pro-domain remains associated with the factor through non-covalent interactions. Two of the factors then form a dimer through two disulfide cysteine linkages. This factor dimer is then stored in the extracellular matrix by binding to the Latent TGF-ß Binding Protein (LTBP) (208). Release of the active (mature) factor is controlled by interactions of LTBP with cell matrix protein thrombospondin or with cell surface integrin proteins (209).

Initiation of the signaling pathway is dependent upon ligand receptor binding. This is mediated through 3 types of receptors. RI and RII receptors are the signaling receptors and are integral transmembrane proteins that exist as dimers on the cell surface (Figure 14,16). Each receptor has a factor binding domain outside the cell and serine-threonine kinase domains inside the cell (213). RIII receptors are passive receptors that do not contribute directly to gene activation. Instead they simply bind free TGF-ß protein and concentrate it at the cell surface (214). Binding of TGF-ß to RII stimulates formation of tetramer and kinase phosphorylation of RI. RII receptor dimer autophosphorylates itself before binding TGF-ß . TGF-ßs bound to the RII dimer now attracts the dimer of RI to the complex (215). The kinase of RII then phosphorylates both RI subunits, which results in activation of the RI kinase. Upon activation, three types of smad proteins then

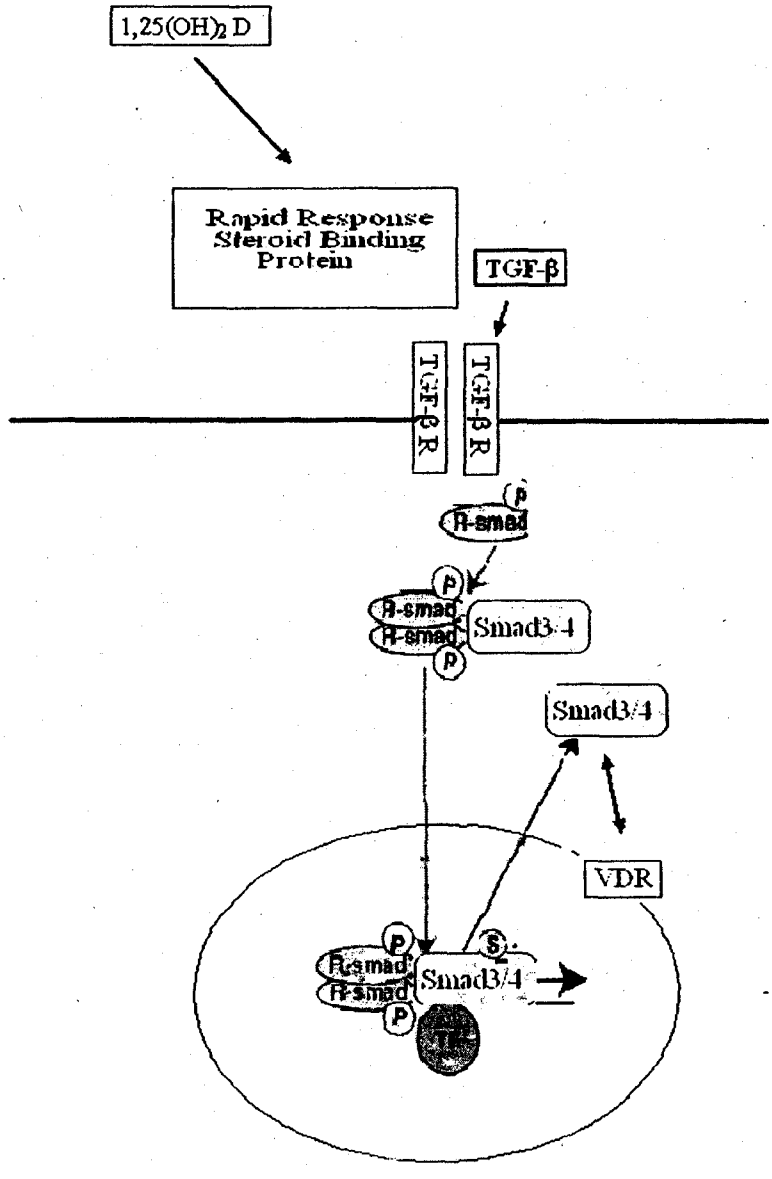



**Figure 16: TGF-ß Signaling Cascade and Multiple Signaling Cascade Interaction with 1,25 dihydroxyvitamin $D_3$** . TGF-ß Signaling is dependent upon ligand receptor binding. This is mediated through 3 types of receptors. Upon activation, three types of smad proteins then function in the TGF Beta signaling pathway; Receptor Regulated Smads (R-Smads), Co-Smads, and Inhibitory Smads (I-Smads). Phosphorylation of $MH_2$ by the RI receptor causes binding of two R-SMADs to one co-SMAD4 (or 4ß) to form a trimeric complex.The NLS then binds the nuclear transport protein importin-ß, causing the entire complex to move into the nucleus . $1,25(OH)_2D_3$ increases TGF-ß synthesis by binding the receptor throught he RRSBP or through smad3 interaction with the VDR itself (216)

function in the TGF Beta signaling pathway; Receptor Regulated Smads (R-Smads), Co-Smads, and Inhibitory Smads (I-Smads). SMADs have two MAD homology domains ($MH_1$ and $MH_2$) joined by a linker sequence. $MH_1$ domain contains the specific DNA binding segment. The $MH_1$ domain also has a nuclear localization signal (NLS) required for the movement of SMAD from the cytoplasm into the nucleus (219,220,221).

When R-SMAD is inactive, the DNA binding site and the NLS are masked by binding with $MH_2$. Phosphorylation of $MH_2$ by the RI receptor causes binding of two R-SMADs to one co-SMAD4 (or 4ß) to form a trimeric complex (217,219). The NLS then binds the nuclear transport protein importin-ß, causing the entire complex to move into the nucleus (218,221). The small GTPase Ran acts to dissociate the importin-ß from the complex, essentially restricting the complex within the nucleus. The SMAD complex then binds to one of the nuclear transcription factors, in this case TFE3, to form a gene specific activator that turns on specific genes, in this case PAI-1 (plasminogen activator inhibitor)(221,222,223). In different cells, different SMAD complexes bind to different nuclear transcription factors, thus activating different genes. Complexes of smad2/smad4 and smad3/smad4 induce expression of protein p15 which arrest the cell cycle in the G1 phase and thereby blocks cell proliferation thereby carrying out the antiprolifarative effects of $1,25(OH)_2D_3$ (224). Furthermore, Smad3 physically associates with VDR and potentiates transcription of *osteopontin* and *osteocalcin* genes in response to TGF-β and vitamin D. $1,25(OH)_2D_3$ interacts with the TGFβ signaling pathway by stimulating signaling through the rapid response steroid binding protein and through an unknown unidentified nuclear component that stabilizes the ligand-dependent complex formation of VDR with Smad3(362). Stimulation with $1,25OH_2D_3$ enhances the association of Smad3 with VDR, heterodimerization of VDR with RXR, and recruitment of the SRC-1/TIF2 co-activator (360,361).

## 1.7.3 Cytokine Signaling Transduction Pathway and Vitamin D

Cytokines are a family of proteins secreted by cells to regulate growth and development in different tissues (228).Cytokines bind and activate specific receptors on target cells causing changes in gene expression to stimulate the development of specific cell types (228). IL-1, IL-6,IL-8 and TNF α are all examples of cytokines which interact with $1,25(OH)_2D$ and stimulate the Janus family protein tyrosine kinase (JAK)(226,227). Furthermore, $1,25(OH)_2D$ interaction with cytokines results in decreased or increased synthesis of proteins, resulting in the anti-proliferative and pro-differentiation characteristics of $1,25(OH)_2D$ which are modulated throught the receptor by a linking protein called the Rapid response steroid binding protein (226.227,228) .

Cytokines share a common structure based on a "four helix bundle" .The four helices are arranged in unique up-up-down-down topology, referred to as the "cytokine fold" (227,228).This topology results in two long loops and one short loop joining the helices. Cytokine receptors come in two formats, simple and complex. Simple receptors have two extracellular cytokine receptor homology (CRH) domains and Complex receptors have the two CRH domains, plus three fibronectin type III repeats and an N-terminal unique domain (227). Inside the cell, both classes are tightly bound to the Janus family protein tyrosine kinase (JAK)(230).

Binding of cytokine to its receptor causes a structural rearrangement of the receptors that moves the two intracellular domains closer together (Figure 14,17). The result of this movement is to move the two JAK-2 enzymes close together, where each

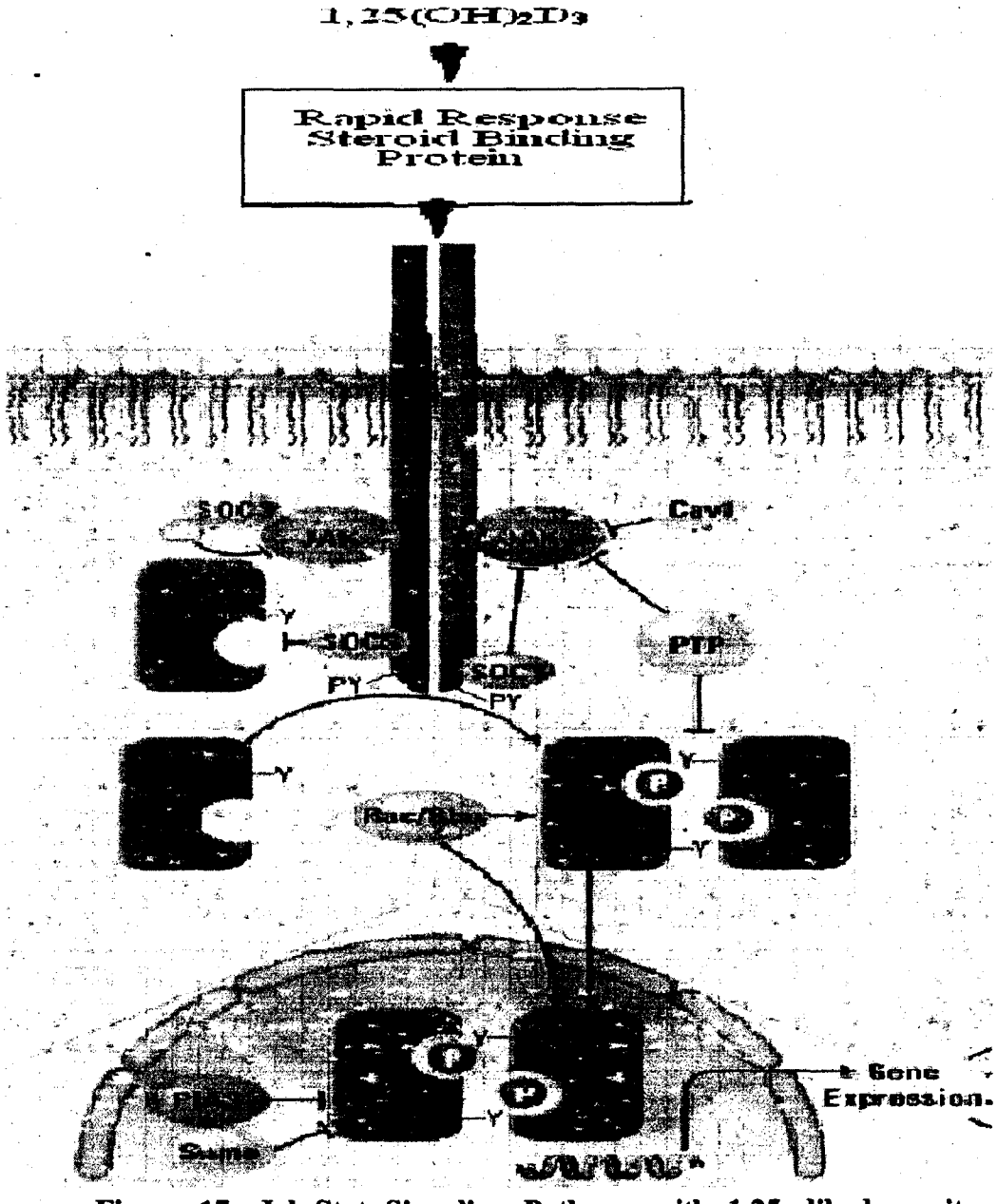



**Figure 17: Jak-Stat Signaling Pathway with 1,25 dihydroxyvitamin $D_3$.** Binding of cytokine to its receptor causes a structural rearrangement of the receptors that moves the two intracellular domains closer together. The result of this movement is to move the two JAK-2 enzymes close together, where each can add a phosphate group to a critical C-terminal tyrosine residue to the other additional tyrosine residues in the intracellular tails of the receptor. Receptor associated JAK kinases activate signal transducers and activators of transcription (STAT) transcription factors bound to a cytokine receptor Upon JAK2 addition of phosphates to tyrosines on the intracellular domain, the SH2 domain of STAT-5 binds to one of these phospho-tyrosines, allowing phosphorylation by the JAK5 enzyme The activated STAT-5 dissociates from the receptor and forms a dimer which then exposes a nuclear localization signal (NLS) which directs the active STAT dimer into the nucleus (232).

can add a phosphate group to a critical C-terminal tyrosine residue to the other additional tyrosine residues in the intracellular tails of the receptor(227). Receptor associated JAK kinases activate signal transducers and activators of transcription (STAT) transcription factors bound to a cytokine receptor (Figure 17) (232). There are 6 different STAT proteins called STAT 1-6. Each binds to different subgroups of cytokinereceptors. Upon JAK2 addition of phosphates to tyrosines on the intracellular domain, the SH2 domain of STAT-5 binds to one of these phospho-tyrosines, allowing phosphorylation by the JAK5 enzyme (233).The activated STAT-5 dissociates from the receptor and forms a dimer. This dimer is stabilized by interactions of the SH2 domains with the C-terminal phospho-tyrosines. The dimer exposes the nuclear localization signal (NLS) which then directs the active STAT dimer into the nucleus where it interacts with other factors to activate genes which induce inhibition of cell growth and promotes differentiation (234). Short term regulation of cytokine receptor signaling is mediated through SHP1 which is a phospho-tyrosine phosphatase. Binding of SHP1 to the receptor activates the phosphatase enzyme which then acts to remove the phosphate from the JAK2 enzyme and inactivates it (233.234). Long term regulation is mediated through Suppressor of Cytokine Signaling Proteins (SOCS)(Figure 17,14)(236)

IL-1α and IL-6 are proinflammatory cytokines, while IL-8 is a proliferative cytokine (224,228). Depending upon which cytokine $1,25(OH)_2D_3$ interacts with, $1,25(OH)_2D_3$ either enhances or diminishes the cytokine synthesis through modulation of the signaling pathway. TNF α ia an example that upon stimulation by $1,25(OH)_2D_3$, $1,25(OH)_2D_3$ induces increased mRNA expression resulting in increased synthesis and the promotion of differentiation (237).Thus, 1,25-dihydroxyvitamn $D_3$ has been shown to improve psoriasis by modulating these cytokines (225).

## 1.7.4 NF-kB Signal Transduction Pathway

Tumor necrosis factor alpha (TNFα) or interleukin-1 (IL-1) production is stimulated by $1,25(OH)_2D_3$ and can induce differentiation and inhibitory growth effects through the JAK–STAT and other pathways (226,227,228,238). Additionally, TNFα or IL-1 activates the cellular protein kinase called TAK1, which in turn activates a second kinase called I-kB kinase (239,240)(Figure 14,17,18). Additionally the NF-kB pathway can also be activated by the secondary pathway phosphatidylinositol 3 kinase which is stimulated by the RAS pathways upon tyrosin kinase activation by $1,25(OH)_2D_3$ (359). The NF-kB is a heterodimer of two subunits, p50 and p65 in resting cells, which is held in the cytoplasm by binding to the inhibitor protein I-kB (Inhibitor of kB)(241). I-kB kinase phosphorylates two serine residues in the N-terminal domains of I-kB. A E3 ubiquitin ligase protein then binds to the phosphorylated I-kB and adds polyubquitin chains. Addition of polyubiquitin causes release of NF-kB from the I-kB inhibitor and immediate degradation of I-kB by the proteosome (Figure 18)(242). Once this occurs, the nuclear localization signals of NF-kB are now exposed, and direct NF-kB into the nucleus where it can bind enhancers and turn on specific regulatory genes (242,243). The NF-kB pathway illustrates how phosphorylation-dependent protein degradation mechanism controls gene expression and its connection to $1,25(OH)_2D_3$ modulation (Figure 14,17,18) (242).

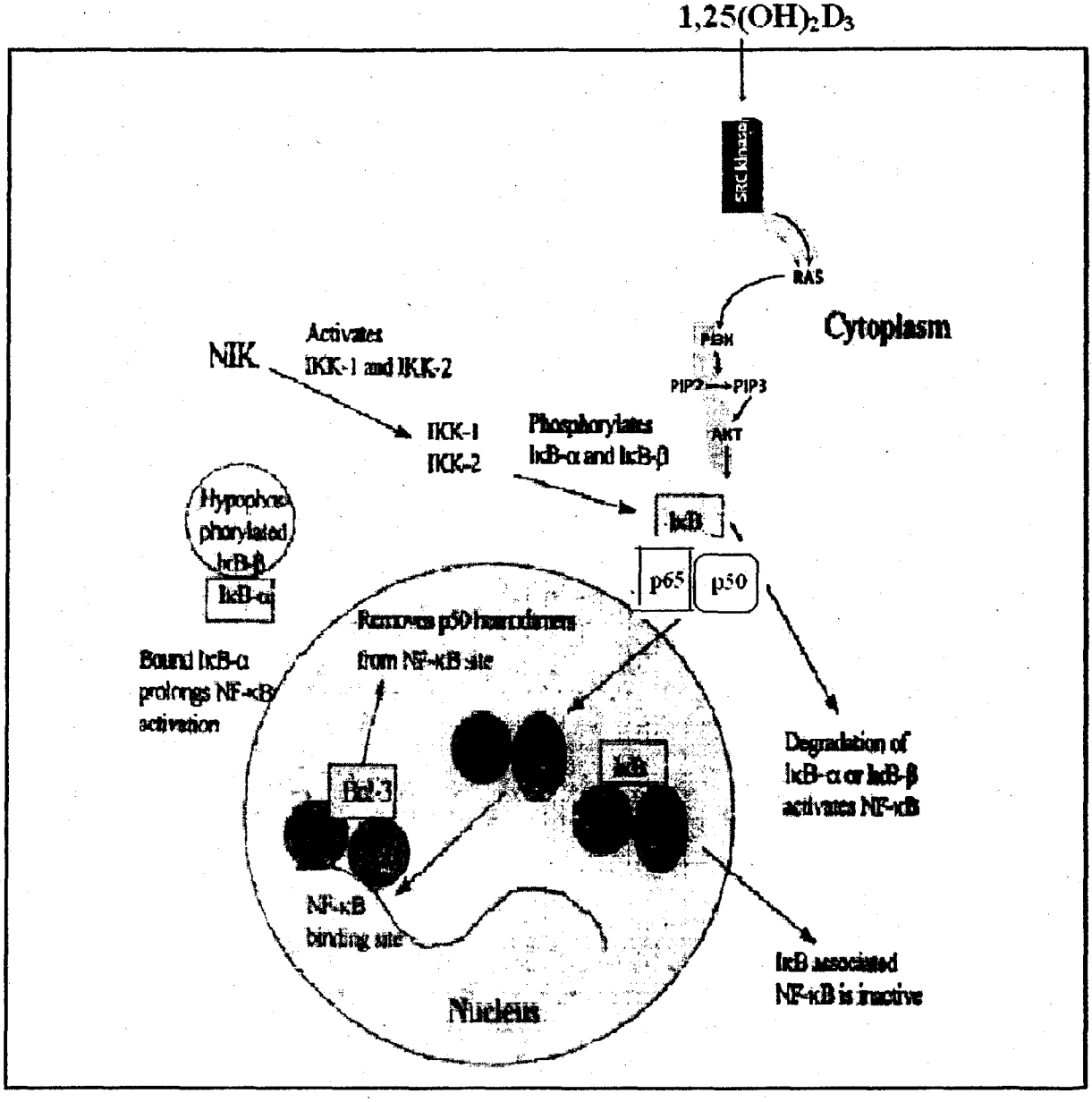



**Figure 18:NFκB Signaling Pathway and Interaction with 1,25 dihydroxyvitamin $D_3$.** The NF-kB pathway can also be activated by the secondary pathway through activation of membrane associated tyrosine kinases which activate the RAS and a multiple of secondary pathways such as phosphatidylinositol 3 kinase which is stimulated by the RAS pathways upon tyrosin kinase activation by $1,25(OH)_2D_3$. The NF-kB pathway illustrates how phosphorylation-dependent protein degradation mechanism controls gene expression and its connection to $1,25(OH)_2D_3$ modulation (242)

## 1.8 Cancer and Oncogenes

Many factors, both intrinsic and extrinsic affect the way cells function within the body. Disruption of cell signaling ultimately affects the expression of genes within the nucleus, which in turn can give rise to cancer (246). Cancer is a multistep process caused by the accumulation of successive mutations within the cell (247). The molecular basis of carcinogenesis is divided into two stages: the initiation stage and the progression stage (248). In the initiation stage a mutation takes place and the mutated cell then replicates in order to progress; this is carried out by promoters which promote the replication of the damaged cell. The continued propagation of the damaged cell leads to the second stage of progression, in which further mutations occur which ultimately result in uncontrolled growth and proliferation leading to tumor formation (248). External factors such as carcinogens, viruses and bacteria also contribute to cancer. Chemical carcinogens and radiation mutagenize DNA while viruses such as Human Papilloma virus (HPV) produce the protein E6 that inactivates p53 causing cancer (247,249,250).

Oncogenes are derived from protooncogenes which are normal genes that when altered become oncogenes causing accelerated and uncontrolled cell growth (250,251). This transformation arises through several mechanisms including point mutations or deletions of DNA. These genetic mutations affect the regulation of cell stages involved in normal cell growth and proliferation (252). Growth factors and cytokines both affect apoptosis and the cell cycle depending on the initiation factor (248). Mutation in the components of the signalling pathways can permanently activate the cell cycle or can block the initiation of cell death ultimately leading to cancer (253). Oncogenes can activate signalling pathways through several mechanisms: 1) excessive amounts of the

signaling molecules involved in growth (248) 2) production of an abnormal receptor that is permanently activated (254) 3) production of abnormal enzymes within the cell resulting in continuous activation of the pathways (255) 4) production of abnormal transcription factors that alter the level of transcription (256). All of the above can ultimately lead to excessive and uncontrolled cell proliferation. $1,25(OH)_2D$ interacts with growth factors,cytokine synthesis and signalling causing inhibition of synthesis of cytokines and growth factors that are proliferative and increasing those that are antiproliferative (186,187,189,190).

## 1.8.1 Mediators of Carcinogenesis; The RAS Oncogene:

The RAS gene is frequently mutated (Figure 14,15) resulting in its activation in 20-30% of cancers and particularly in 30% of breast cancer (257). Mutated RAS genes have been detected in 95% of pancreatic carcinomas, 67% of human leukemia's, 54% of head and neck tumours and 60% of hepatocellular carcinomas (258-262). It is easily transformed from a protooncogenes to an oncogene by a single point mutation in its coding sequence (263). Mutations have been localized to codons 12,13,59 and 61 (261,262). However, carcinogenesis cannot occur solely by activation of the RAS gene, but in synergy with other oncogenes such as c-Myc, n-Myc and E1A which are all nuclear transcription factors and activate cell proliferation (254,262).

Three 21 Kda RAS genes have been identified in the mammalian genome, designated as H-ras-1,K-ras-2 and N-Ras (265). Their locations on human chromosomes have been located. N-RAS has been assigned to the short arm of the human chromosome 1 (1p22-p32) whereas H-RAS-1 and K-Ras-2 have been located on the short arm of chromosome 11 and 12 (263). The three RAS genes code for a closely related protein

known as p21. Other members of the RAS superfamily include RHO, RAL and RAP (264).

RAS is a monomeric GTP-binding switch protein that alternates between the active and inactive states (263). It is synthesized in the cytosol and become associated with the inner side of the plasma membrane after posttranslational modifications. It is normally found in the inactive form and it is activated through the conversion of GDP to GTP (268). Once activated it activates the signalling cascade that sends signals to the nucleus to activate transcription of genes required for cell cycle progression. GTP hydrolysis removes the GTP from the RAS and resets it to its inactive form (267,268). When RAS is mutated, GTP hydrolysis is blocked and RAS is then constantly activated and sends continuous signals to the nucleus through the MAPK pathway. The proteins required for cell cycle progression are continuously expressed resulting in uncontrolled cell proliferation (264,268).

The MAPK pathway can also be independently activated by other means then by RAS and can lead to cell transformation and ultimately tumor formation (269). This has been demonstrated in renal carcinoma in which the MAPK pathway is continuously active independent of RAS (270). Over expression of any component of the signalling pathway such as Raf, MEK, MAPK results in transformation and oncogenesis (268,269,270). Experimental evidence indicates that MAPK inhibitors can inhibit oncogenic cell growth (271).

## 1.8.2 Cyclins and Cycle Dependent Kinases

Cyclins and cycle dependent kinases (CDK) work together to guide the cell through the check points of the cell cycle (Figure 14)(272). They are tightly regulated to ensure the smooth and timely transitions from G1 to S or G2 to M. Cycle dependent

Kinases inhibitors (CDKI) act to repress the cyclin/CDK blocking the cell cycle. The CDKI correspond to each set of cyclin/CDKs and inhibit the cell cycle progression at each stage (273). It is a well orchestrated system comprised of several cyclin dependent kinases with corresponding cyclins that associate and act at the different stages of the cell cycle. Oncogenes can affect the regulation of the cell cycle in two manners: 1) They can over expression the cyclins and CDK continuously driving the cell cycle and 2 ) they can inhibit the CDKI which are then unable to block CDK/cyclin complexes resulting in continuous progression through the cell cycle (274,275).1,25$(OH)_2D_3$ has been shown to decrease cyclin gene expression and to inhibit cell cycle progression (273-276).

## 1.8.3 Tumor Suppressor Genes: Retinoblastoma (Rb)

The Retinoblastoma (Rb) gene is located on chromosome 13q14 and is a tumor suppressor gene that is a central regulator of proliferation (Figure 14) (349). Loss of both of these alleles results in cell proliferation and tumor formation (350). Rb binds to a transcription factor called E2F which is responsible for the transcription of S phase genes. E2F is inactivated when it binds Rb in the unphosphorylated state. Hyperphosphorylation of Rb releases E2F which then stimulate transcription of *S* phase genes (351). The cyclins continuously drive the cell cycle by causing the hyperphosphorylations of Rb subsequently releasing E2F from Rb stimulating the transcription of *S* phase genes. Consequently, 1,25$(OH)_2D_3$ decreases cyclin gene expression causes Rb to remain in its unphosphorylated state thereby inhibiting transcription of *S* phase genes (351) .

## 1.8.4 p53: Mediator of Apoptosis

p53 is one of the most important genes involved in the cell cycle because it is responsible for initiating the DNA repair mechanisms (Figure 14). Half of all human cancer is characterized by a loss of the p53 gene and particularly in more advanced

carcinomas (277). The gene is located on chromosome 17 and responds to different types of cell stress (278). When the cell is exposed to carcinogens, mutagens, radiation and cytotoxic compounds p53 transcription is stimulated. P53 hen stops the cell cycle arresting the cell in G1 and allows the cell to undergo cell repair (279). Its action is mediated through the up regulation of genes that produce proteins such p21 (CDK cell cycle Inhibitor) and GADD45 a protein involved in DNA repair (280). If the damage to the cell is too extensive then p53 stimulates the BAX gene which then induces apoptosis (281). If the cell is damaged and this regulation does not occur, the cell cycle would then continue to replicate with damaged DNA. This will cause further mutations eventually resulting in the formation of cancer cells (282).

## 1.9 Breast Cancer Models

Mammary tumorigenesis arises in a stepwise fashion involving the acquisition of distinct genetic perturbations which contributes to full malignancy (243). Genetically, these alterations are grouped into two groups: 1) gain of function and 2) loss of function mutations. Gain of function mutations occur in protooncogenes while loss of function mutations generally occurs in tumor suppressor genes (247,250,251). In order to study breast carcinogenesis and the effects of mutations on key components of signaling pathways the utilization of human derived cancer cell lines have been established (285,286). Yet these *in vitro* models may not reflect the *in vivo* biology. Consequently, *in vivo* mice models are used to mimic human diseases (284). In order to study breast cancer; animal models of premalignant disease, specifically designated to mimic early, intermediate and late stage of tumor progression have been created (285,288). Presently, several models of mammary oncogenesis are available including chemical carcinogenesis, cardio injection of cancerous cells and a multitude of transgenic models

such as MMTV/*neu*, Her2/*neu*, MMTV-*c-myc*, MMTV-Ha-*RAS* and MMTV-ErbB2 (358,359,360). Three models for studying breast cancer specifically utilizing the Polyoma Middle T-Antigen are: 1) viral infection of neonatal mice 2) viral infection of adult immunoincompetent mice (athymic Balb/c *nu/nu* nude mice, SCID mice) and 3) transgenic mouse lines that express a viral oncoprotein such as large T or middle T antigen under the control of a mouse mammary tumor virus (MMTV) promoter (286).

## 1.9.1 Viral Infection of Neonatal Mice

In order to simulate breast cancer utilizing the Polyoma Middle T-Antigen without using a transgenic approach, newborn mice are injected with the Polyoma Middle T-Antigen one week after birth. Infection of neonatal mice with the viral protein leads to rapid systemic infection that involves large number of tissues (290). Tissue distribution depends upon the viral strain used and the route of infection. Typically the major organs infected are the bone, skin, kidneys, liver, lungs, and mammary glands (291). The viral burden peak occurs between 8-10 days post infection which then declines due to an antiviral response (292). Tumors appearance and distribution depends upon which virus in association with which mouse strain is used (293). Tumor density and appearance correlates with viral replication potential and areas in which viral replication can be easily facilitated.

## 1.9.2 Immuno-Incompetent(Athymic/SCID) Mouse Infection Model

Immuno-incompetent adult mice is a model based upon the neonatel mouse model but used to study the induction of tumors at a specific stage that by passes the antiviral effects of the immune system that is associated with the neonatal model (292). The neonatal model is lethal by one month of age due to the massive infection that it succumbs too. In order to study breast cancer progression, the adult model is employed

due to its restriction to three tissues being the skin, bone and mammary gland (294,295). In this model, two weeks post-injection with the Polyoma Middle T-Antigen is associated with hyperplasia followed by ductal dysplasias (295). Overt tumors are observed by 4-6 weeks post injection and are adenocarcinomas of ductal origin. Age at the time of infection is extremely important and has a drastic impact on tumor incidence and the number of tumors per infected mouse (292,294).

### 1.9.3 Transgenic Mice: Polyoma Middle T-Antigen Mouse Mammary Tumor Virus Strain 634

Mouse mammary tumor virus (MMTV) is an archetypal B-type retrovirus whose presence is linked to the development of mammary adenocarcinomas (296). The MMTV does not contain a conventional oncogene and therefore the induction of mammary adenocarcinomas by MMTV is closely tied to the activation of cellular proto-oncogenes such as neu , Polyomavirus (PyV) large and middle T antigens by insertional mutagenic activation (297). The Long terminal repeats (LTR) of MMTV can activate nearby genes through steroid hormone dependent mechanisms. The tumors are restricted to the mammary epithelium because they are under the control of the mammary gland specific MMTV promoter/enhancer (298).

Polyomavirus (PyV) is a member of the polyomavirinae family which include primate simian virus 40 (SV40) and infects a variety of vertebrate species (296). Polyomavirinae family is a small non-enveloped double stranded DNA virus with icosahedral capsids which have tremendous ability to induce tumors in inbred mice. Polyomavirus produces three proteins from mRNA, which are generated by transcribing their genome: Large-T antigen, Middle T-antigen and Small T-antigen (297). The three T-antigens are activated by the same promoter which transcribes a single mRNA that is

alternatively spliced resulting in the three T-antigens. T-antigens interact with specific cellular regulatory proteins, acting as repressors or stimulators of the cell cycle and subversion of the function of these regulatory proteins result in unrestricted cell growth (298). Mice models use both Large and Middle T-antigens as protooncogenes to induce mammary adenocarcinomas (297).

Polyoma middle T antigen mouse mammary tumor virus strain 634 is a transgenic mouse model strain that is used to simulate breast cancer and its progression ultimately leading to insights into its signaling pathways and their mode of action (299).Breast cancer is associated with increased signaling activity in pathways such as RAS-MAPK, TGFβ, NfκB and secondary pathways such as phosphatidylinositol-3-kinase, IP3 and DAG (300). The transforming activity of middle T-antigen is dependent with its association with a number of cellular proteins. It acts as a potent oncogene because it binds to and co-opts signal transduction pathways, including those of the *Src*, RAS and *PI3* kinase family pathways (Figure19)(297).

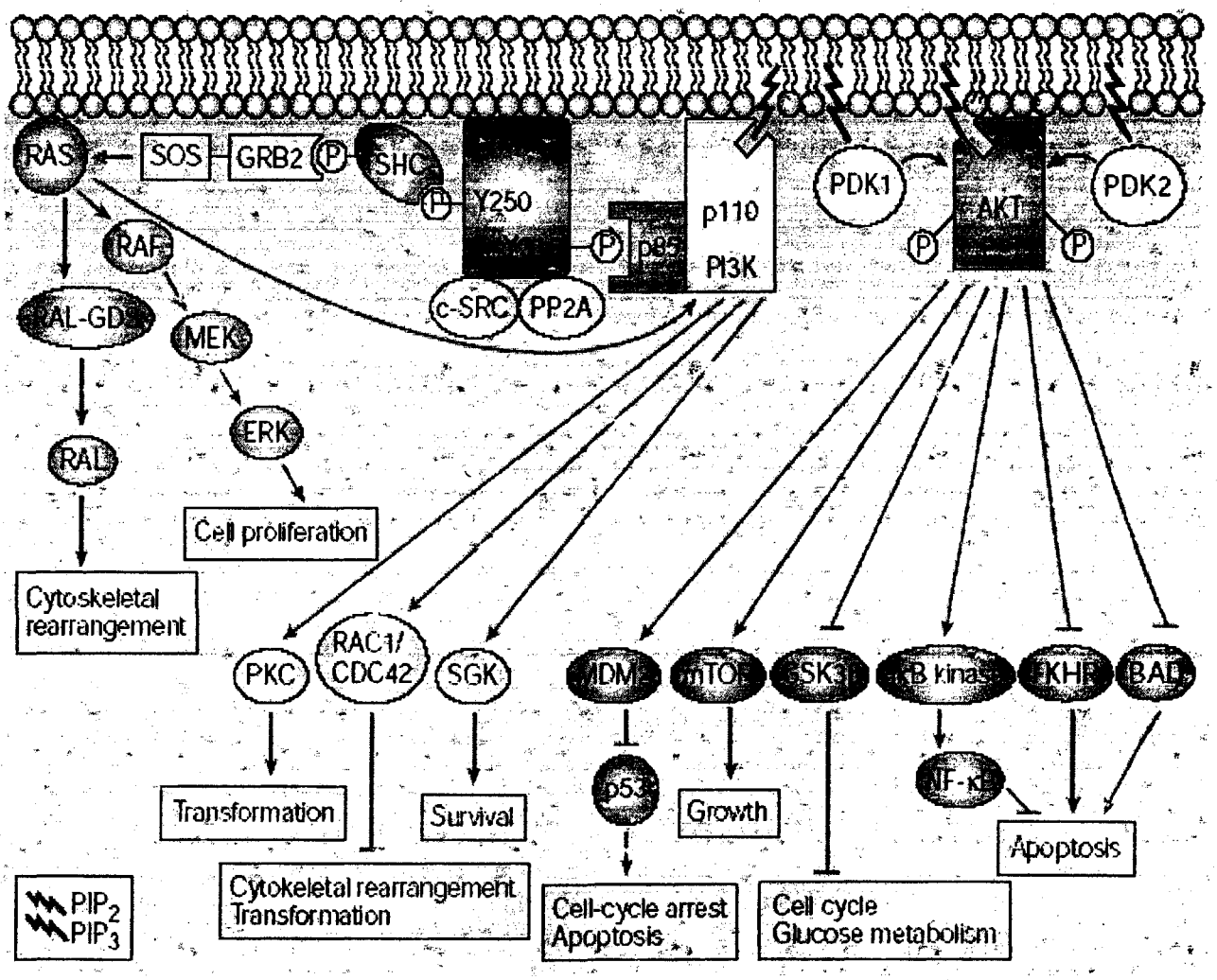



**Figure 19: Polyoma Middle T-Antigen Mouse Mammary Tumor Virus interaction with Different Signaling Pathways.** Membrane Located Middle T antigens binds and activates the different signaling cascades through binding of a phosphotyrosin of SHC that then through use of its $SH_2$ domain stimulates the different pathways (286). (See text for details)

MAPK and PI3K cascade are initiated when membrane located middle T antigen binds protein phosphatase 2A and C-*src* which causing the phosphorylation of tyrosine residues in the carboxy-terminal end of middle T-antigen. This provides the docking for the $SH_2$ domains of GRB2 which then links to SOS7 and results in the activation of RAS/MAPK pathways and other pathways involved in phosphorylation of tyrosin residues (305). Middle T antigen tyrosin residue 315 binds to the $SH_2$ domain of PI3K activating the kinase activity and resulting in the activation of the various components ultimately affecting AKT which controls apoptosis (306).

In the PyVMT transgenic model of breast cancer all female PyVMT rapidly develop multifocal mammary adenocarcinomas that are palpable, as early as 5-7 weeks of age (290-292). In addition, female PyMT mice also develop pulmonary metastases by ~3-4 months of age, with an extremely high penetrance (~90-100%) (291). Importantly, this mouse tumor model has been shown to recapitulate human breast cancer progression, from early hyperplasia to malignant breast carcinoma, including the up regulation of ErbB2/Neu and cyclin D1 expression (303). It is therefore an optimal model for breast cancer as it mimics the actual cascade events that coincide with the disease (Figure19,20).

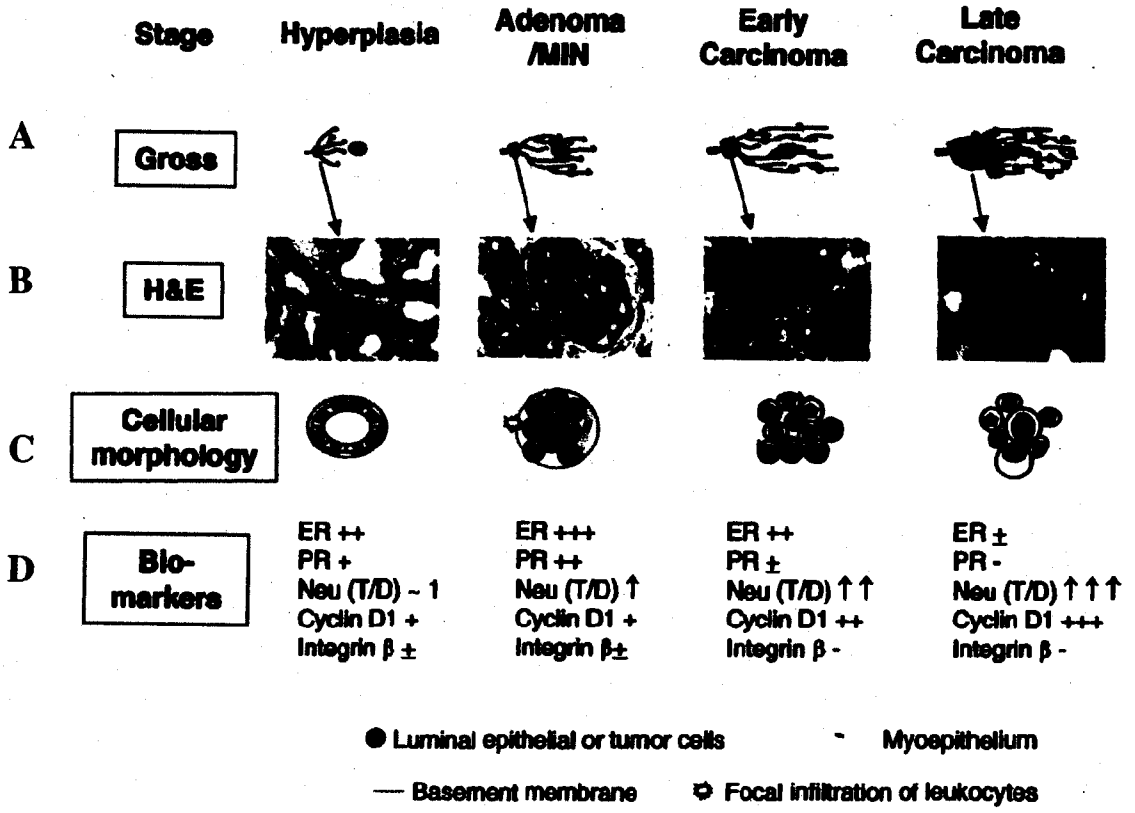



**Figure 20: Summary of Tumor Progression and Biomarker Expression in PyMT Mouse model of Breast Cancer.** **(A)** Overall development of lesions in mammary glands of PyVMT mice. Tumor lesions are indicated by blue dots. **(B)** H&E staining corresponding histology of primary lesions at different stages of tumor progression. **(C)** Cellular morphology panel schematically illustrates changes in the cytology of the cells **(D)** Changes in biomarkers during tumor progression (318).

## 1.10 Vitamin D and Breast Cancer

1,25-dihydroxyvitamn D is involved in the regulation of many organ systems and tissues (4,5). It is not only a key regulator of calcium homeostasis but also plays a central roles in the synthesis of cytokines and growth factors and uses their transduction signaling pathways to regulate growth, development, angiogenesis, metastasis, cell cycle regulators, oncogenes, tumor suppressor genes and apoptosis (Figure10,14).

Optimal circulating concentrations of 25OHD are between 50-90ng/mL and concentrations consistently below 30-20 ng/mL are associated with vitamin D deficiency and rickets (Table 1)(4). Vitamin D is important for the maintenance of bone health and neuromuscular function (11).Vitamin D deficiency has been implicated in the development of multiple sclerosis, cardio vascular disease, type 1 diabetes mellitus, kidney disease, autoimmune diseases and specifically cancer (307). Several studies point to the importance of vitamin D in a variety of cancers including colon, ovarian, prostate oesophageal, non-Hodgkin's lymphoma and breast cancer (308).The connection between cancer and vitamin D has been made through correlation and distribution of cancer in comparison to geographic location and sunshine exposure (Table 2). Vitamin D and 25OHD concentrations are inversely correlated with latitude and increase with sun exposure (309,310,311). It has been documented that higher latitudes are associated with increased cases of cancer and especially above $37^0$ N and below 37 ° S of latitude which result in the decreased synthesis of vitamin $D_3$ (312). Other factors which diminish vitamin $D_3$ production by the skin can be associated with reduced UVB penetrance of the skin due to skin pigmentation (melanin), clothing, sun screen and urbanization (313).

**Table 2: Variation of Serum 25OHD levels with Season and Latitude (11)**

| Location | Latitude | Population age range(y) | Summer/Fall High,SD (ng/mL) | Summer/Fall High,SD (nmol/L) | Winter/Spring Low,SD (ng/mL) | Winter/Spring Low,SD (nmol/L) |
|---|---|---|---|---|---|---|
| Miami, Florida | 26° N | Men and Women >18 | 26.8 ±10.3 (males) 25.0± 9.4 (females) | 67.0±20.6 (males) 62.5 ±23.5 (females) | 23.3±8.4 | 58.25± 21 |
| United states overall | | African American Women | 19.8 | 49.5 | 15.5 | 38.75 |
| | | Caucasian women | 36.4 | 91 | 26.4 | 66.0 |
| Omaha Nebraska | 41.3° N | Elderly women | 34.2 ± 2.0 | 85.5±5.0 | 27.4±2.7 | 68.5±6.75 |
| Framingham, Massachusetts | 42.5° N | Men 67-95 | 39.1 | 97.75 | 31.6 | 79.0 |
| | | Women 67-95 | 31.6 | 79 | 24.4 | 61.0 |
| Boston, Massachusetts | 43.3° N | African American Women 20-40 | 16.4±6.6 | 41± 16.5 | 12.1±7.9 | 30.25± 19.75 |
| | | Caucasian women 20-40 | 34.2 ±13.2 | 85.5 ±33 | 24.0±8.6 | 60± 21.5 |
| Toronto, Ontario | 43.7° N | Young women | 30.4± 11.2 | 76 ± 28 | 23.2±9.6 | 58 ± 24.0 |
| Portland, Oregon | 45.5° N | Men and women | 24.7 ±8.0 | 61.75 ± 20 | 20.4±7.6 | 51± 19.0 |
| Paris France | 49° N | Adolescent males | 23.4 ±8.0 | 58.5± 20 | 8.2±2.8 | 20.5± 7.0 |
| Calgary, Alberta | 51° N | Men and women 27-89 | 28.6 ±9.4 | 71.5± 23.5 | 22.9±8.5 | 57.25 ±21.25 |

Epidemiological evidence links low levels of vitamin D to increased risk of cancer (315,316). African American participants in an epidemiological study living in cities have the lowest concentrations of vitamin D and the highest risk of cancer. This is likely due to decrease absorbance of UVB light due to the colour pigmentation of their skin as well as decreased sun exposure in urbanized centers. An inverse correlation also exists between exposure to UVB radiation and the development of breast cancer (Figure20) (314,315). Furthermore, increased circulating concentrations of vitamin D and its metabolite 25OHD are associated with lower risk of breast cancer whereas low concentrations are associated with faster progression of metastatic breast cancer (314). Similarly *in vivo* administration of $1,25(OH)_2D_3$ or its analogs shows reduced mammary cancer in mice and rats (152,333,334).Furthermore, in various *in vitro* models such as the keratinocyte HPK1A,HPK1A RAS and MCF-7 cell lines which mimic breast cancer, vitamin D has been shown to inhibit proliferation and promote differentiation (316-320,333-338) .

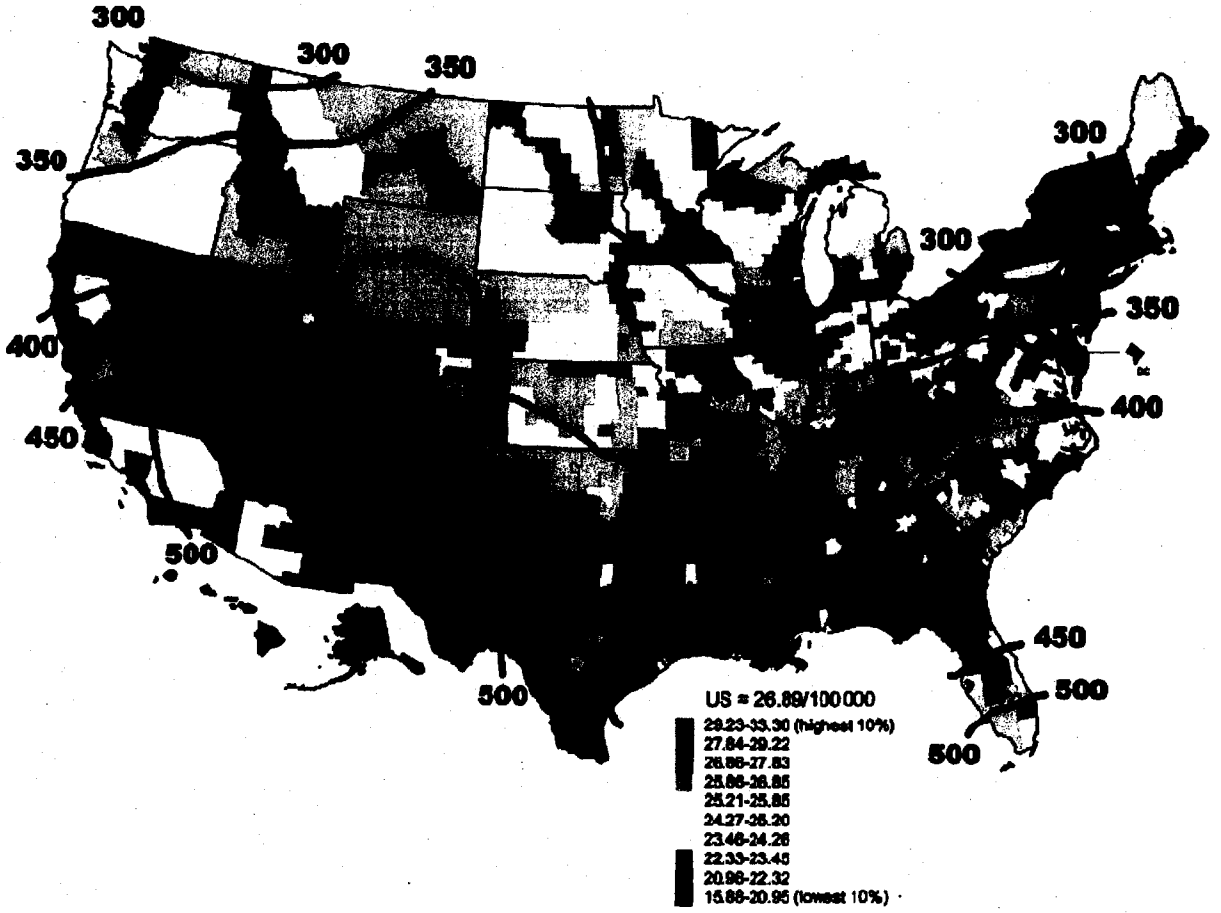



**Figure 21: Breast Cancer Mortality Rates by Country Area and Mean Daily Solar Irradiance**. The blue areas of the graph indicate high daily solar irradiance and low breast cancer mortality The white areas indicate medium areas of mortality and median daily solar irradiance. The red areas indicate high areas of mortality with low daily solar irradiance (11).

## 1.11 Rational

Important functions of $1,25(OH)_2D$ have been recognized and include suppression of cell growth, induction of cell differentiation and immunosuppresion (316-320). As a result $1,25(OH)_2D$ has been seen as a potential treatment for a variety of disorders including psoriasis, cancer and immunological diseases (110,224,235,317).But due to the hypercalcemic side effects of $1,25(OH)_2D_3$ and its analogs, its utilization has been curtailed. Dr. Kremer's laboratory has confirmed in both human keratinocytes and in melanoma cells that the extra-renal production of $1,25(OH)_2D_3$ exerts principally if not exclusively a local autocrine function (304,317). This and the fact that the kidney is not the unique site of $1,25(OH)_2D_3$ production in the body raises the question if administration of inactive vitamin D precursors can reduce tumor growth *in vivo* with no visible side effects such as hypercalcemia (17,50,56).

## 1.12 Hypothesis

The sterol hormone 1,25-dihydroxyvitamn $D_3$ ($1,25(OH)_2D_3$ ) is well known for its actions on calcium and phosphate homeostasis, which in turn regulates bone mineralization (4). $1,25(OH)_2D_3$ the active form of vitamin D, is also a potent inhibitor of tumor growth *in vitro* and *in vivo* (316-320). Furthermore, the kidney is not the sole location and production site of the 1αhydroxylase enzyme and it has been shown that $1,25(OH)_2D_3$ can be produced by other tissues including keratinocytes and mammary epithelial cells (17,50,56,318). Thus, raising the possibility that local production of $1,25(OH)_2D_3$ by breast cancer cells may play a role in tumor growth and progression. We therefore hypothesize that local conversion of $25OHD_3$ to$1,25(OH)_2D_3$ in breast tumor cells inhibit breast tumor growth and progression.

## 1.13 Objectives of This Project

A. Analysis of tumor growth and progression: Animals treated with a constant infusion of 1,25-dihydroxyvitamn $D_3$ ($1,25(OH)_2D_3$) (12pM/24h),its inactive precursor, $25OHD_3$ (2000 pM/24h) or vehicle will be compared.

B. Tumor onset, *in situ* epithelial hyperplasia, adenocarcinomas and lung metastases will be determined at timed intervals and compared between groups.

C. Specific biomarkers of tumor progression will be used as indicators of tumor progression: ER-α, Erb2/Neu,Cyclin D1 , Integrin-β1 and Ki-67

D. Finally the mechanism(s) by which locally produced $1,25(OH)_2D_3$ inhibits tumor progression will be examined .

# Chapter 2: Material & Methods

In this chapter, I will describe the material and procedures that I followed to achieve the objectives of this study

## 2.1 Animal Studies: Mice and Histology

All procedures involving mice were conducted in accordance with the Canadian institute of Health Research regulations, the National Institutes of Health regulations and the consensus report recommendations from the Annapolis Meeting concerning the use and care of experimental animals (285). The study of mice was approved by the McGill University Animal Care committee, (Montreal Quebec, Canada). All animals were housed and maintained in a barrier facility at the Royal Victoria Hospital Institute for Animal Studies, McGill University. Mice were kept on a 12-hour/dark cycle with *ad libitum* access to chow (Picolab 20, PMI Nutrition International) and water. MMTV-Polyoma middle T antigen (PyMT)transgenic mice (strain #634) were generated as previously described .Briefly,PyVMT MMTV #634 breast cancer mice expressing were obtained from

Dr. William Muller (McGill University) on an FVB background (***Fv1***$^{b}$ allele for sensitivity to the B strain of Friend leukemia inbred genetic background). Male homozygous PyVMT mice were randomly bred with FVB females lacking the PyVMT transgene to obtain female mice heterozygous for the PyVMT transgene. These were then further crossed to obtain homozygous female PyVMT MMTV 634. All mice analyzed in this study were homozygous for the PyVMT transgene on FVB background.

Random selections of mammary tumor carcinomas were used for whole mount preparation. Mammary tumors were excised, cut into smaller portions, and fixed with 10% neutral buffered formalin for over 24 hours at 4°C and then transferred to cold 70% ethanol or PBS before embedding in paraffin .Tissues Sections were cut at 5 microns and paraffin-embedded, sectioned, and stained with haematoxylin and eosin (H&E). Analyses and descriptions were performed in accordance with the guidelines put forth by the mouse mammary gland pathology consensus meeting in Annapolis.

## 2.2 Pump Implantation

Animals were treated with vehicle or a constant infusion of $1,25(OH)_2D_3$ (12pM/h) or the vitamin D precursor, $25OHD_3$ (2000pM/24h).Osmotic minipumps (model 2004 Alzet; Alza Corporation,Palo Alto, CA) were implanted subcutaneously under light Anesthesia (Xylazine, Ketamine,Aceprumazine and 0 .9%NaCl) at 4 weeks of age. Each minipump contained either $1,25(OH)_2D_3$ or $25OHD_3$ or vehicle dissolved in 1 ml of propylene glycol, ( 200 µl of ethanol, and 800 µl of saline) to deliver a continuous dose of the compound for up to 28 days at a delivery rate of 0.25ul/hour.At 8 weeks of age pumps were reimplanted and continuous dose of the compound were delivered until sacrifice at 12 weeks.

## 2.3 Tumor Palpation and Excision

Female mice were palpated three times a week beginning at 4 weeks of age for the development of tumors in the mammary gland. Mice were examined in a genotype-blinded fashion and palpated in each of the ten mammary glands up until 12 weeks of age.3D tumor measurements were done using calipers. Tumor diameter long axis (L) and mean mid-axis width (W) were measured to estimate the tumor volume using the following formula:

$$V = \frac{4}{3}\pi\left(\frac{L}{2}\frac{W}{2}\right)$$

Growth curves were generated by plotting the mean tumor volume beginning at 4 weeks, female mice were sacrificed before tumor diameters reached 1.5 cm as required by the animal protocol. All mammary tumors were carefully excised and weighed. Portions of the tumors were also frozen at -80° C or stored in formalin for fixation purposes.

## 2.4 Measurement of Blood Parameters &Body Weight

Animals were bled once a week for measurement of total plasma calcium, phosphorus, albumin, alkaline phosphatase and creatinine. Plasma calcium, Phosphorus, albumin, alkaline phosphatase and creatinine levels were determined by microchemistry (McGill University, McIntyre Animal Center, Montreal Quebec, Canada). Corrected plasma calcium was calculated using the formula: plasma total calcium + [(40 - plasma albumin)] x 0.02. Body weight was measured using an LS200 balance (OHAUS, Switzerland). These measurements were carried out at 9:00 a.m. once a week from the beginning of the experiments.

## 2.5 Measurements of $1,25(OH)_2D$ and 25OHD Levels in Tissues & Blood:

At sacrifice blood was collected for analysis of $1,25(OH)_2D$ and 25OHD. Tumors and kidney were extracted in 70% ethanol and centrifuged for 10 minutes at 10000g. Aliquots of 70% ethanol containing $1,25(OH)_2D$ and 25OHD were then sent to Dr.Horst (USDA, Agriculture Research) for quantification.

## 2.6 Lung Metastasis Analysis

Female PyVMT mice at 12 weeks of age were sacrificed and the lungs exposed by thoracic and tracheal dissection. Before removal, lungs were injected with 2 mL of 10% neutral buffered formalin by tracheal cannulation in order to fix the inner air spaces and inflate the lung lobes. Lungs were then excised and placed into formalin for 48 hours. Nine lung lobes were separated in order to visualize all surfaces of each lobe. During counting, an equal number of lobes were matched for size and location in the thorax. Representative lungs were also paraffin-embedded and processed for histological analysis. Care was taken so that any grossly evident metastases that were dissected during sectioning were only represented once in the subsequent haematoxylin and eosin stained slides. Surface lung metastases were scored in a genotype-blinded fashion under low power using a Nikon SMZ-1500 stereomicroscope. The total tissue area (metastases and normal lung tissue) was calculated using software provided by the Center for Bone & Periodontal Research (Montreal Quebec). After this had been completed for all slides from all the specimens, each slide was then examined again and areas of metastatic tissue were calculated. The total area of metastatic tissue were then subtracted from the total surface area and compared between groups. Subsequent calculations (ie, number and percent metastases) were based on these initial data calculations.

## 2.7 Immunohistochemistry (IHC)

To identify cells expressing, ER-α, Erb2/Neu, and Integrin-β1,formalin-fixed mammary gland sections were immunostained using rabbit polyclonal antibodies raised against a peptide mapping at the N-terminus of ER-α, the human Neu gp185, and the extracellular domain of human Integrin-β1 (sc-538, sc-284,and sc-8978) respectively (Santa Cruz Biotechnology Inc.,Santa Cruz, CA). To identify cells expressing cyclin D1, mammary gland sections were immunostained using antibody raised against a mouse monoclonal antibody raised against a recombinant fusion protein of mouse origin (Sc-450) (Santa Cruz Biotechnology Inc.,Santa Cruz, CA). To identify cells expressing Ki-67, affinity purified goat polyclonal antibody raised against a peptide mapping near the C-terminus of Ki-67 of mouse origin was used. To identify 1αhydroxylase (1 alpha(OH)ase/ CYP27B1) protein expression levels in tumor cells, sheep immunoglobulin IgG fraction sheep anti-murin-25-HyhydroxyvitaminD3-1–alpha hydroxylase antibody was used(Binding Site, PC290). Secondary antibodies used were either donkey anti Goat (Sc-2020,Santa Cruz Biotechnology Inc., Santa Cruz, CA),Binding site AP360, Binding site Colorado California), goat anti-mouse (Sc-2005),and goat anti-rabbit(Sc-2004) were used in association with its primary antibody counterpart. Specific reactivity was detected using a peroxidase-based detection kit (Vector Laboratories, Burlingame, CA). As negative controls, adjacent sections were processed without the primary antibody. ER-α, Erb2/Neu, Integrin-β1, Cyclin D1 Ki-67, and 1α(OH)ase positive cells were scored by visual examination of 21 randomly selected fields at X 400 magnification containing at least 500 cells.

## 2.8 Western Blotting &Analysis

150 to 200 mg portions of tumor tissue frozen in liquid nitrogen were homogenized using a polytron tissue grinder in 2 mL of RIPA buffer at 4°C consisting of 1% Nonidet P-40, 1% sodiumdodecyl sulfate, 0.5% sodium dexocholate, protease inhibitor cocktail (Sigma Chemical Co.), and phosphate buffered saline buffer (PH 7.0) for 5 and 2 minutes, respectively. Tissue lysates were then centrifuged at 12,000 x g for 10 min to remove insoluble debris. Protein concentrations were analyzed using the Beckman photospectromoter 640 using an optical density at 260 nm. HPK1A and RAS extracts were prepared by using a whole cell extraction methods .Briefly, cells were grown to 70% confluencey in Dulbeccos modified Eagle medium (DMEM)(Invitrogen, Ontario, Canada) in 10%FBS and then harvested following trypsinization. Whole cell lysates were colleted using the freeze thaw protocol and total protein concentration was quantified by Beckman photospectrometer 640 .Tissue or cell lysates were separated by SDS-PAGE (8-12% acrylamide) and transferred to nitrocellulose membrane. The nitrocellulose membranes were treated with wash buffers containing 10 mM Tris pH 8.0, 150 mM NaCl,0.05% Tween-20 (TBS-Tween), supplemented with 1% bovine serum albumin (BSA) and 4% nonfat dry milk (Carnation) for the blocking solution and 1% BSA for the antibody diluents. Primary antibodies were used at a 1:1000dilution. Horseradishperoxidase (HRP)-conjugated secondary antibodies (anti-mouse 1:5,000 dilution (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were used to visualize bound primary antibodies, with the chemiluminescence substrate (Santa Cruz Biotechnology Inc.,Santa Cruz, CA).Western blot gel preparation and immunoblotting were performed following standard procedures.

## 2.9 Cell Culture

HPK1A and HPK1A RAS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, and antibiotic/mycotic (Invitrogen, Ontario, Canada)

## 2.10 RNA Extraction

Tumor tissues preserved at -80°C at time of sacrifice were washed with DEPC-treated water and homogenized on ice using 1ml of 100mM NaCL,10mm Tris pH 7.5 1mM EDTA..5%Np-40 and a protease inhibitor cocktail (Roche Applied Bioscience). Lysates were spun in a nicrofuge for 30 minutes (10000 g, 4C Biofuge).Before RNA extraction, tumor lysates were treated with RNaseout (Invitrogen, Ontario, Canada) for 5 minutes at room temperature. Total RNA was extracted from the tumor lysates using Trizol LS (Invitrogen, Ontario, Canada) according to the procedure suggested by the manufacturer. Purified RNA was quantified by spectrophotometry using an optical density at 260 nm.

## 2.11 RT-PCR

Sense primer 5'TCTATGAGCTT TCCCGGCACCCC-'3 and anti-sense primer 5' TCAGGTAGCTCTTCAAAATGGGTCAA-'3, were used to quantify 1αOHase levels of expression. RT-PCR was done according to the procedure suggested by the manufacturer (Fermentas RT-PCR kit).RT-PCR products were visualized on a 1% agarose gel using the Gel Doc system (Bio-Rad 4.4 GEL Doc) and using the Bio-Rad quantity one software version 4.4.1.

## 2.12 Statistical Analysis

All results are expressed as mean ± SE. Statistical comparisons for in vitro study were made using the unpaired Student's t test (a probability value of $P < 0.05$ was considered significant). The statistical difference of tumor on set rate of the animals was determined by Kaplan-Meier analysis. The number of animals used in this experiment was determined based upon the availability and quantity of animals required to have statistically significant findings .Twelve animals per group was determined based upon availability, statistical significance and the relationship between the amount of tissue needed for the predetermined required experiments which were calculated to correlated the number of animals required to produce the necessary amounts of tissue needed for experimentation .

# Chapter 3 Results

## 3.1 Identification and Quantification of Expression of The 1-Alpha Hydroxylase Enzyme in Polyoma Middle T-Antigen Mouse Mammary Tumor Virus(MMTV) Strain 634 Mice .

The 1αOHase enzyme belongs to the cytochrome p450 enzyme superfamily and is mainly expressed in the proximal tubules of the kidneys (57). In addition to the kidneys, 1αOHase enzyme has been localized in various tissues of the body including skin, brain, breast, testis, bone, small intestine blood and recently in several cancer cell lines such as the human keratinocytes models of HPK1A,HPK1A*ras*, human breast cancer cells such as MCF-7 and in melanoma cells (17,50,56). Thus, this raises the possibility that local production of $1,25(OH)_2D$ by breast cancer cells may play a role in tumor growth and progression. Hence, our first task was to confirm the presence of the 1αOHase enzyme in our PyVMT-MMTV model of mammary tumorigenesis. Immunohistochemistry (IHC) was done using IgG fraction sheep anti-murin $25\text{-}OHD_3$-1αOHase antibody (PC290, Binding Site CO, California,) on PyVMT MMTV tumors. The keratinocytes cell line HPK1ARAS was used as a positive control. (Figure 22A-F,Figure34 E).Additionally, we used normal breast tissues of the same mouse strain to determine basal physiological levels of 1αOHase in breast tissues (Figure 22A-F, Figure 35 E).The 1αOHase enzyme was identified by IHC in the nucleus of mammary tumors, normal breast and HPK1ARAS cells. As a negative control we used tissue sections stained without the primary antibody. Thus, using IHC we identified 1αOHase in both normal breast and in tumor cells suggesting that this local expression may potentially influence tumor growth and progression by facilitating the conversion of $25OHD_3$ to $1,25(OH)_2D_3$ which then

potentially controls tumoral progression and proliferation through an autocrine mechanism.

Mammary tumors develop in 100% of PyVMT mice by 7-8 weeks of age (290-292). Four stages of tumor progression have been described in the PyVMT MMTV634 mouse model: hyperplasia, adenoma/mammary intraepithelial neoplasia (MIN), and early and late carcinoma (318). The hyperplasia stage is characterized by cluster (s) of densely packed lobules formed on the duct that connects to the main milk-collecting duct while at the late carcinoma stage tumors are composed of solid sheets of epithelial cells with little or no remaining acinar structures visible (Figure 20) (318,322). In order to analyze expression of the 1αOHase enzyme and whether this expression changed during tumor progression we quantified 1αOHase by western blotting and RT-PCR in normal breast tissue and in tumor tissues at all stages of tumor progression: hyperplasia (premalignant lesion 5-7 weeks of age), MIN/Adenoma (8-9weeks) and late carcinoma stage (10-13 weeks of age) (Figure 22E,F). As indicated, expression levels of the 1αOHase were not increased or decreased in normal mouse breast or at any of the different stages of tumor progression but remained at a constant level. Thus demonstrating that tumor progression does not effect the expression of the 1αOHase enzyme or the local conversion of $25OHD_3$ to $1,25(OH)_2D_3$ .

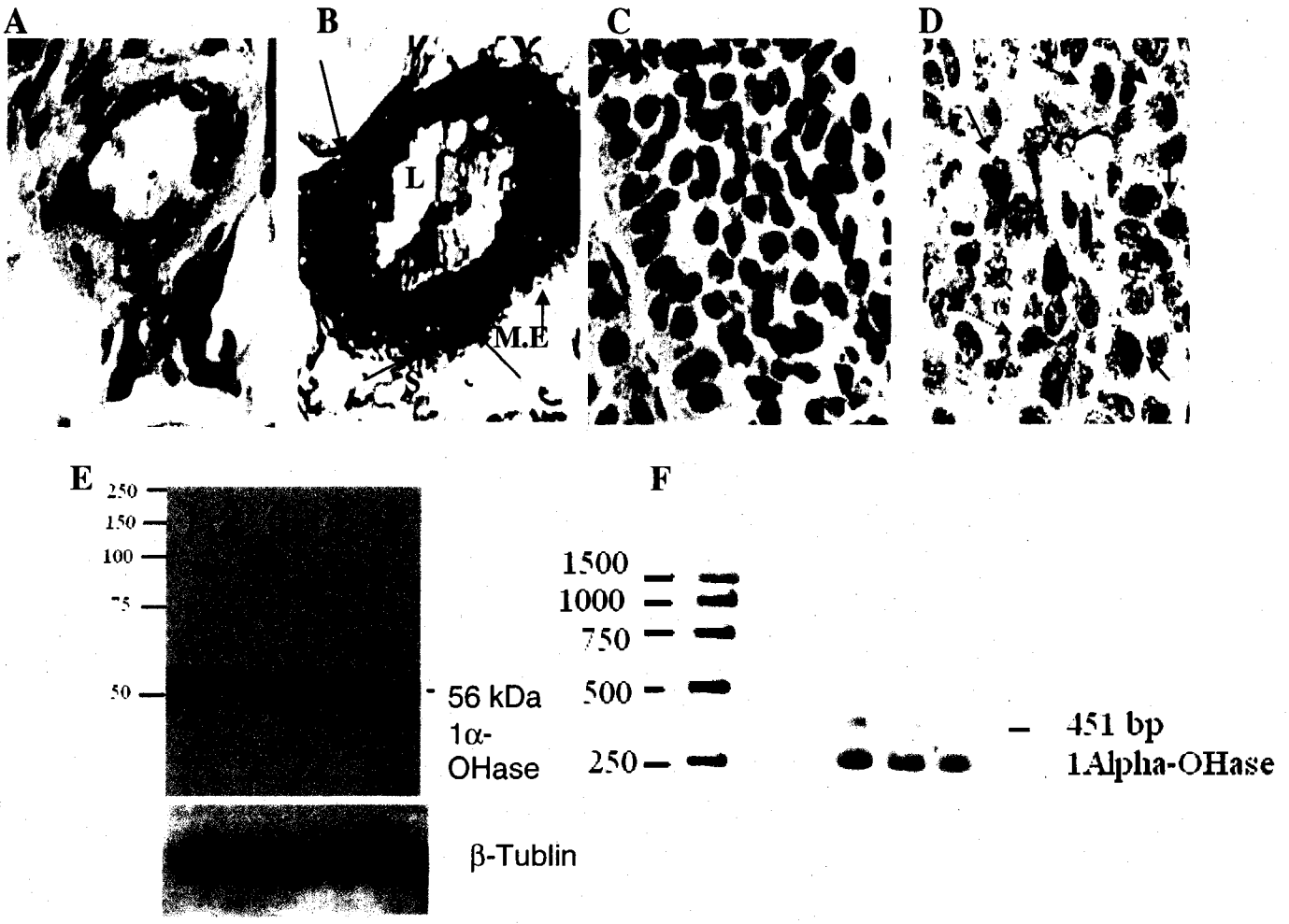



**Figure 22. Expression of 1αhydroxylase (1αOHase) in Normal and Tumor Tissues. Upper Panels (A-D): Immunhistochemistry**. Formalin-fixed mammary gland sections were immunostained with a sheep antimurine 1αOHase antibody (The Binding Site, San Diego, California). Specific reactivity was detected using a peroxidase-based detection kit. Arrows indicate areas of positive staining. (**A**) Normal breast control staining tissue section (**B**) Normal breast expression of 1αOHase staining tissue section. Arrows are indicative of the presence of myoepithelial cells. Myoepithelial cells is abbreviate in the above figure by M.E , S for Stromal Cells and L for Luminal Cells (**C**) PyVMT mammary tumor of 11-12 week old animal control staining section (**D**) PyVMT mammary tumor of 11-12 week old animal 1αOHase expression. Arrows indicate of the expression of the 1αOHase in mammary tumour epithelial cells of (**E**) Western Blot : Lane 1 Normal FVB Breast Tissue. Lane 2 HPK1A RAS keratinocyte cell line (positive control). Lane 3 PyVMT MMTV 634 Tumor 5-7 weeks old. Lane 4 PyVMT MMTV 634 Tumor weeks8-9 weeks old. Lane 5 PyVMT MMTV 634 Tumor 11-12 weeks old (**F**). RT-PCR of 451 bp1αOHase gene utilizing the primer sequences: sense 5'TCTATGAGCTTTCCCGGCACCCC-'3 and antisense 5'TCAGGTAGCTCTTCAAA ATGGGTCAA-'3.Lane 1 Normal FVB Breast Tissue.Lane 2 HPK1A RAS keratinocyte cell line (positive control). Lane 3 PyVMT MMTV 634 Tumor 5-7 weeks old. Lane 4 PyVMT MMTV 634 Tumor weeks8-9 weeks old. Lane 5 PyVMT MMTV 634 Tumor 11-12 weeks old. The first band represent the 1αOHase expression .The second band represent excess DNTP's

## 3.2 Effects of the inactive precursor $25OHD_3$ on Tumor onset & Tumor Number as Compared to Positive ($1,25(OH)_2D_3$) and Negative controls (Vehicle)

PyVMT MMTV 634 begins to develop palpable tumors on average between 5-6 weeks of age. By 6-7 weeks of age, 100% of animals had palpable tumors as observed previously (290-292,318). The effects of $25OHD_3$ on tumor onset and progression was examined *in vivo* following subcutaneous implantation with mini osmotic pumps at a constant infusion rate of 2000 pM/24h. Another group of animals were infused with $1,25(OH)_2D_3$ (12pM/24h) and the last group with vehicle only. The number of mice that developed tumors over time were analyzed longitudinally using Kaplan-Meier analysis (Figure 23).Animals treated with $25OHD_3$ had a significant delay in tumor onset as compared to the untreated group. At seven weeks of age, 100 percent of vehicle treated mice had palpable tumors. 44% of $1.25(OH)_2D_3$ treated mice had palpable tumors at 7 weeks ($P<.002$)(Figure 22A).In contrast, only 25% of $25OHD_3$ treated mice had a palpable tumor at 7 weeks of age ($P<.002$)(Figure 23B). $25OHD_3$ and $1,25(OH)_2D_3$ secondary tumor appearance was also significantly delayed in both treated groups (Figure 23C,D) as compared to vehicle treated group. 100% of vehicle treated mice had a palpable secondary tumor at 9 weeks of age as compared to only 10% in the $1,25(OH)_2D_3$ (Figure 23 C) ($P<.002$) and none in the $25OHD_3$ treated groups (Figure 23D).

We next analyzed the number of tumors per animal in the vitamin D treated groups as compared to the untreated control group (Figure 25 A, Table 3).At sacrifice the number of tumors was markedly lowered in vitamin D treated animals as compared to the untreated group. A reduction of 66.6% in tumor number with a corresponding mean of

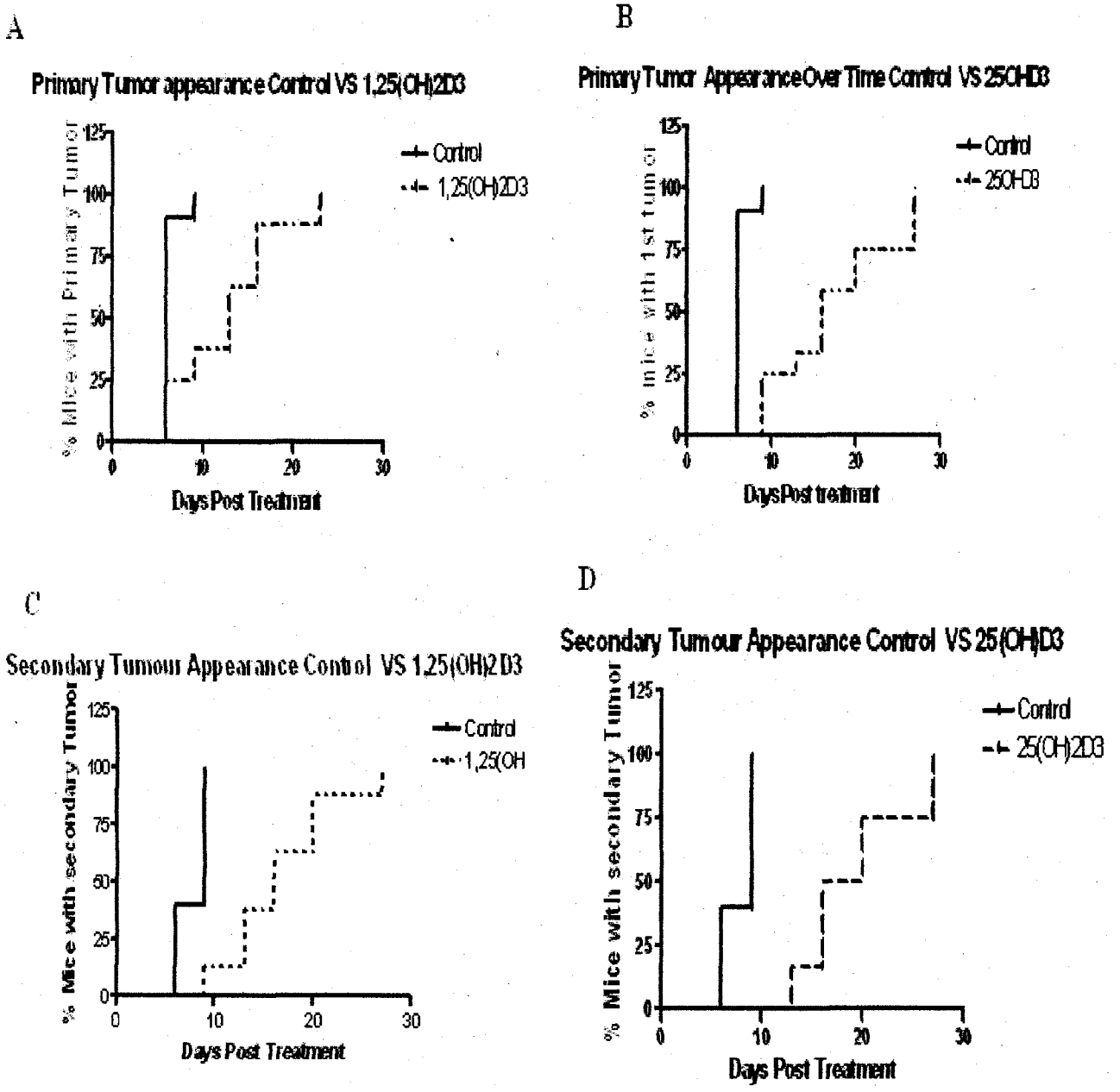



**Figure 23. Effect of $25OHD_3$ and $1,25(OH)_2D_3$ as Compared to Vehicle on Tumor Onset of Primary and Secondary Tumors.** Kaplan-Meier analysis of breast tumor occurrence in PyVMT female transgenic mice. Mice were examined three times per week. Log rank tests of survival plots of the date indicate a statistically significant difference between either $25OHD_3$ (n=12) or $1,25(OH)_2D_3$(n=9) treated animals as compared to vehicle treated animals (n=10) ($p<0.002$). The above tumor percent onset is measure at the time interval at which 100% of the vehicle control group had palpable mammary tumors in comparison to the $1,25(OH)_2D_3$ and $25OHD_3$ groups.

**Table 3: Tumor Number and Percentage of Control with Primary & Secondary Tumors**

| | | Animals with Primary Tumor (%) | Animals with Secondary Tumor (%) | Average Tumor Number per Mouse per group |
|---|---|---|---|---|
| **Untreated** | **(n=10)** | **100** | **100** | **4.8 ±1.39** |
| **$1,25(OH)_2D_3$** | **(n=9)** | **44*** | **10*** | **3.22 ±1.39*** |
| **$25OHD_3$** | **(n=12)** | **25*** | **0*** | **3.20 ±1.39*** |
| | **p** | **.002** | **.002** | **.01** |

**A P value of <0.05 was considered significant**

**Table 3: Tumor Number and Percentage of Control Animals in Comparison to treated groups with Primary & Secondary Tumors.** Animals were treated with vehicle or a constant infusion of $1,25(OH)_2D_3$ (12pM/h) or the vitamin D precursor, $25OHD_3$ (2000pM/24h) for a period of approximately 60 days. Female mice were palpated 3 times a week beginning at 4 weeks of age for the development of tumors in the mammary gland. Mice were examined in a genotype-blinded fashion and palpated in each of the ten mammary glands until 12 weeks of age. Primary and secondary tumor appearance percentages were calculate upon detection of primary and secondary tumors in all of the vehicle control mice in comparison to the $25OHD_3$ (2000pM/24h) and $1,25(OH)_2D_3$ (12pM/h) treated groups.

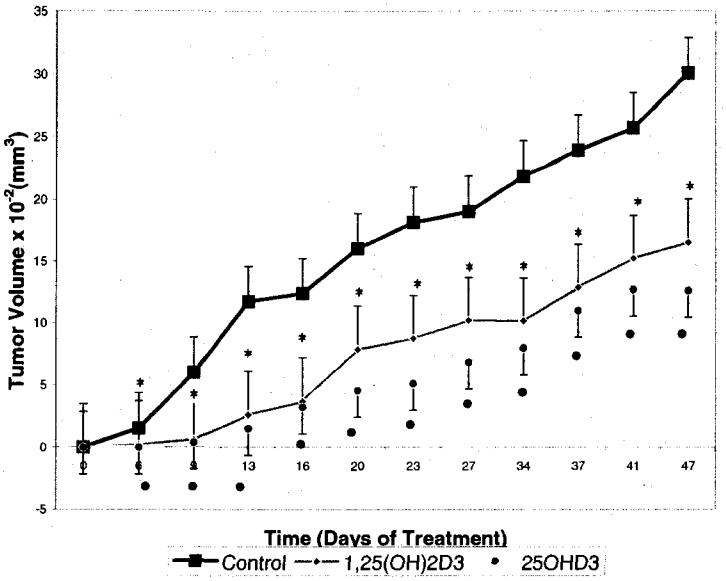
3.20±.96 tumors in the $25OHD_3$ treated group and a reduction of 67% with a corresponding mean of 3.22±1.09 in the $1,25(OH)_2D_3$ treated-group as compared to 4.8 ±1.39 tumors in vehicle treated animals

## 3.3 Effects of $25OHD_3$ and $1,25(OH)_2D_3$ on Tumor Growth

The efficacy of $25OHD_3$ and $1.25(OH)_2D_3$ on tumor growth is shown in figure 24A and figure 25A-C . In the control group, tumors grew rapidly reaching a volume of 1817.9±142. $mm^3$ at 23 days and 3008.±285 $mm^3$ by 47 days during the treatment period, whereas the tumor volume remained significantly lower (P<.002)(Figure 24A Figure 25B) throughout the course of the experiments in both $25OHD_3$ and $1,25(OH)_2D_3$ treated animals. At 23 days $25OHD_3$ and $1,25(OH)_2D_3$ treated mice had an average tumor volume of 576.79±153.70$mm^3$ and 874±145. P<.002) respectively; at 47 days $25OHD_3$ and $1,25(OH)_2D_3$ had an average tumor volume of group of 1260± 216 $mm^3$ and 1654±25 (P<.002). Overall the mean reduction in tumor volume at sacrifice in $25OHD_3$ animals was 58±2.1% (P<.002) and 45± 2.5%(P<.002) in $25OHD_3$ and $1,25(OH)_2D_3$ treated animals. Tumors in the untreated control group (Figure 25 B,C.) were not restricted to the upper breast but were also found and equally distributed in all of the 10 mammary glands (Figure 25 A). In contrast $25OHD_3$ and $1,25(OH)_2D_3$ treated animals has tumors in only 3.2±0.8 glands and 5.12±0.3 respectively (Figure 25A-C).

The mean weight of mice in the control group increased by 56 ±0.7% (Figure 24B) as compared to 26.8±0.8 % (P<.002) in the $25OHD_3$ and 35.3±.3% (P<.05) in the $1,25(OH)_2D_3$ treated group (Figure 24 B). At sacrifice, a reduction in tumor weight was observed but was not found to be significant.

A




B

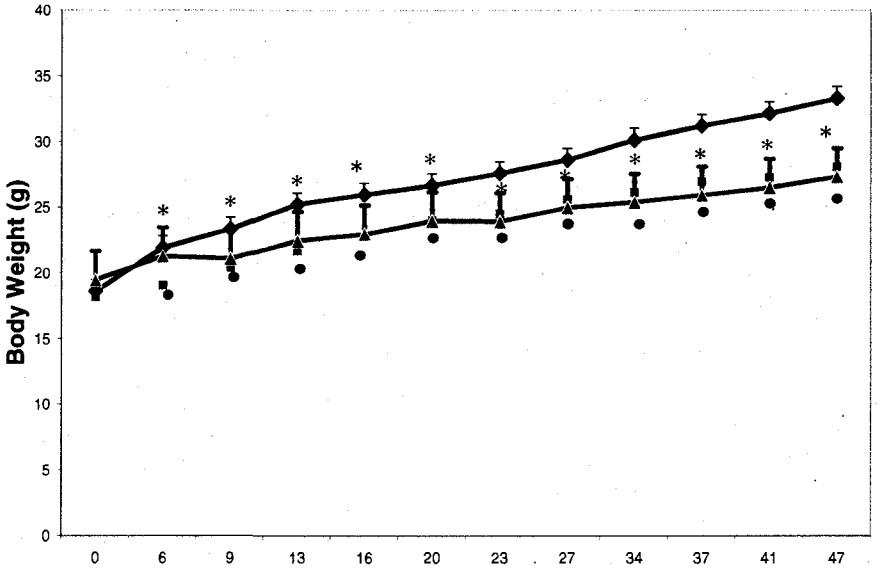



**Figure 24: Tumour Growth Kinetics in PyVMT Mice: (A)** $25OHD_3$ (2000 pM/24h), $1,25(OH)_2D_3$ (12 pM/24h) or vehicle were administered by constant infusion using Alzet osmotic mini-pump. Data are expressed as means ±SD of the number of animals in each group. Number of animals analyzed for $250HD_3$ (n=12), $1,25(OH)_2D_3$(n=9) untreated (n=10). * indicates significant difference from vehicle treated animals at each time point ($P$<.002). Note the significant reduction in tumour growth in $250HD_3$ treated and $1,25(OH)_2D_3$ treated animals as compared to vehicle treated animals. The high standard deviation at days 6 and 9 in relation to tumor size is explained by the fact that each member of each group experience a different rate of tumor on set and growth with in the group itself which is represented by the large standard deviation at these time points and the Kaplan-Meier analysis of tumor onset (Figure 23)(**B**) The mice groups were weighted twice a week. $250HD_3$ and $1,25(OH)_2D_3$ treated groups had a significant increase in total body weight that equally corresponded with tumor volume $1,25(OH)_2D_3$ (P<.05) $250HD_3$ (P<.002). * represents a significant difference between the $1,25(OH)_2D_3$ and the control while • represents a significant difference between $250HD_3$ and the control.

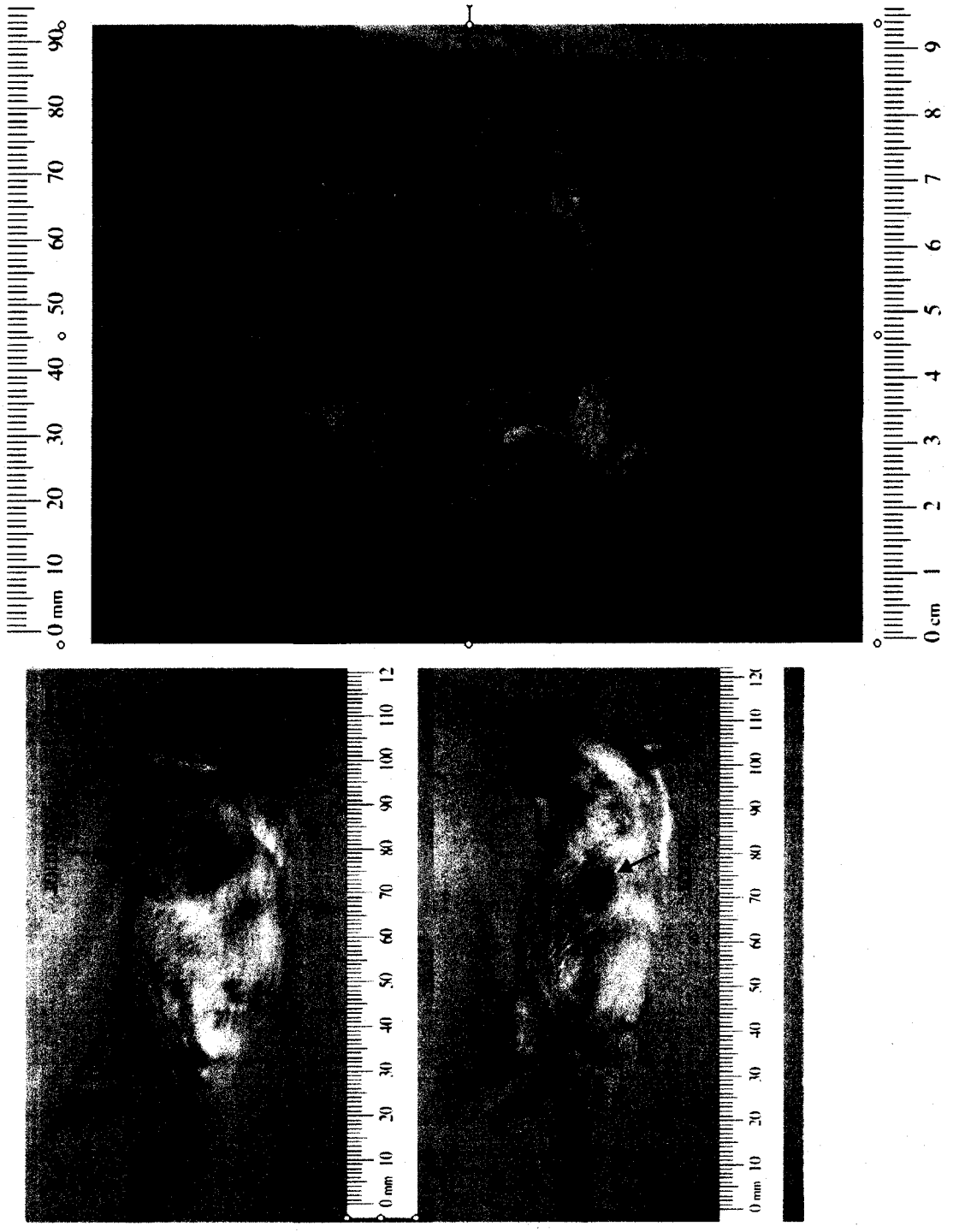


**Figure 25: Tumor Size at Sacrifice**: **(A)** Tumor size at sacrifice were measured and compared between groups. **(B)** The untreated vehicle control group **(C)** $25OHD_3$ treated group.

## 3.4 Effects of 1,25-dihydroxyvitamin$D_3$ and 25O$HD_3$ on Tumor Histology

To investigate the effect of 25O$HD_3$ and 1,25$(OH)_2D_3$ on the histology of PyVMT MMTV 634 mammary tumors, tumor sections obtained at sacrifice were stained with H&E. As shown in Figure 26, the histological appearance of tumors from mice treated with 25O$HD_3$ and 1,25$(OH)_2D_3$ is different from that of the vehicle treated control group (Figure 26 A-C).Tumor sections from the vehicle treated group were composed of solid sheets of epithelial cells with drastically diminished and barely visible acinar structures (Figure 26 A, D). In comparison tumors from the 25O$HD_3$ and 1,25$(OH)_2D_3$ treated groups consisted of clusters of well defined densely packed lobules with ornate epithelial proliferation still confined by a basement membrane and connective tissue with minimal cytological atypia (Figure 26 B,C,E,F,G). Additionally, greater cytological atypia was observed in the untreated group in comparison to 25O$HD_3$ and 1,25$(OH)_2D_3$ (Figure 26 D-F).The cells that comprised these sheets of tumor cells in vehicle treated animals had a wide range of variability in cellular and nuclear size demonstrating pleiomorphic variation in nuclear morphology, size and shape (Figure 26 D).Conversely, 25O$HD_3$ and 1,25$(OH)_2D_3$ treated group had cytological acini also completely filled with solid sheets but with relatively uniform cells with round morphology but not possess pleiomorphic features as observed with the untreated group (Figure 26 A-F). In addition to increased nuclear pleomorphism group, the border of individual tumor acini in these areas became unclear in the untreated control group comparison to the 25O$HD_3$ and 1,25$(OH)_2D_3$ treated groups . Multiple tumor nodules were observed throughout the mammary glands of all three groups but were significantly reduced in both the 25O$HD_3$ and 1,25$(OH)_2D_3$ treated animals (Figure 26A-C).

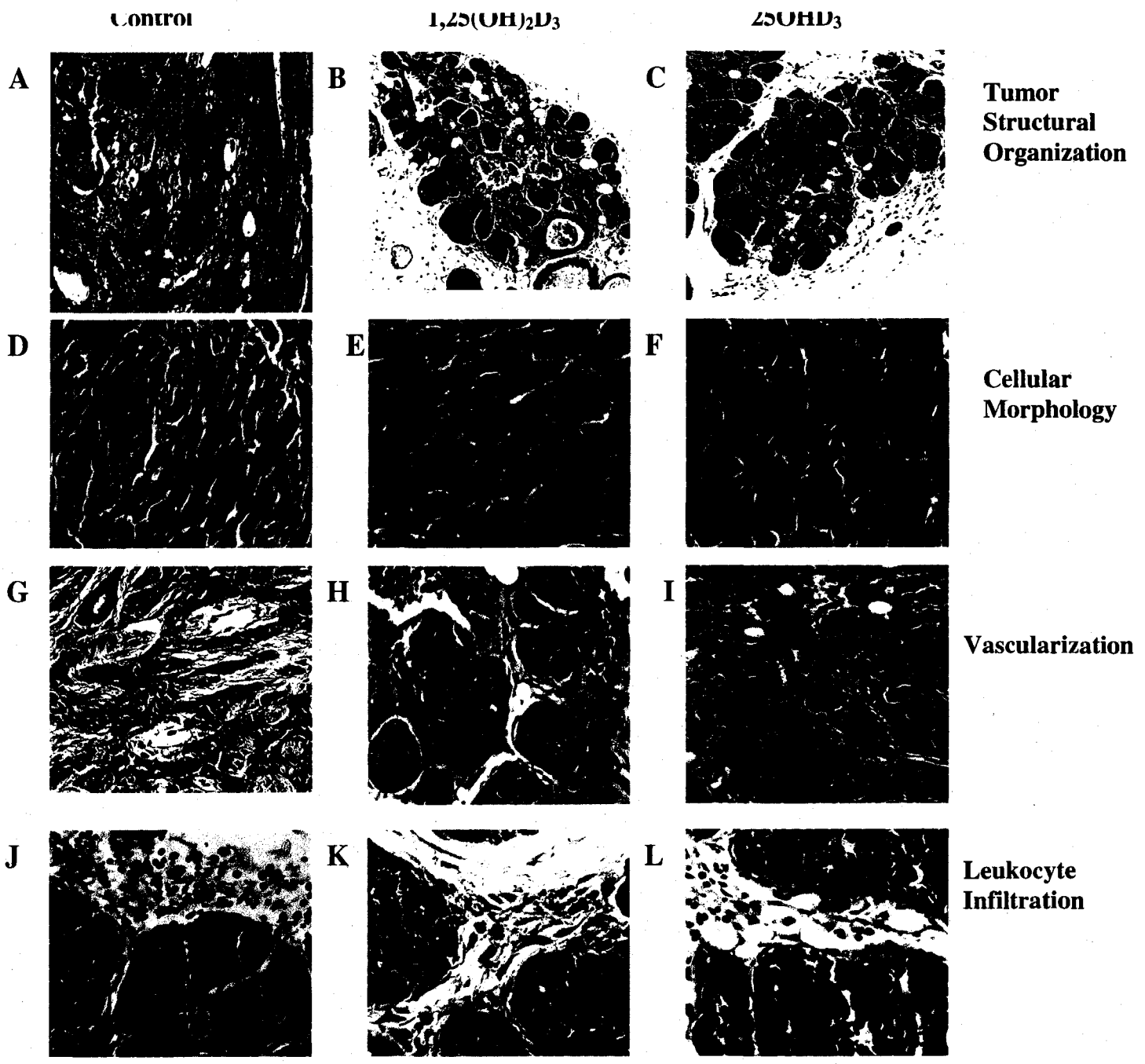



**Figure 26: Effect of $25OHD_3$ and $1,25(OH)_2D_3$ on the Tumor Histological Appearance of PyVMT Female Transgenic Mice.** Representative images of tumor sections stained with H&E were captured through a computer attached to a microscope. **A** ,**D**,**G**, and **J** are tumors from untreated animals; **B**,**E**,**H** and **K** are tumors from animals treated with $1,25(OH)_2D_3$; **C**,**F**,**I** and **L** are tumors from animals treated with $25OHD_3$ for 47 days. Figure A-C illustrate the morphological and structural difference between the $25OHD_3$ and $1,25(OH)_2D_3$ treated groups in comparison to the untreated group. Figure D-F show an increase in cytological atypia and pleiomorphic variation in nuclear morphology, size and shape in the untreated group in comparison to $25OHD_3$ and $1,25(OH)_2D_3$. Figure G-I reveals the increased vascularization in the untreated group in comparison to the $25OHD_3$ and $1,25(OH)_2D_3$ groups. Figure J-L exhibits the increased Leukocyte infiltration in the untreated group in comparison to the $25OHD_3$ and $1,25(OH)_2D_3$ Magnification, 100x for A-C and 400X for D-L.

Markedly increased vascularity was also observed throughout all tumor sections in the untreated control group as compared to tumor sections from both $25OHD_3$ and $1,25(OH)_2D_3$ treated groups (Figure 26A-C,G-I). Tumors from the control group had a high density of foci leukocytes infiltration in the vicinity of the tumor in the surrounding stroma (Figure 26 B,C J-L). These infiltrates were composed of cells with the morphology of macrophages, fibrolasts and neutrophils. The majority of these infiltrating cells were of mononuclear appearance with macrophage morphology; cells resembling granulocytes were also observed (Figure 26J). $25OHD_3$ and $1,25(OH)_2D_3$ tumor sections also had leukocytes infiltration in areas adjacent to tumor nodules (Figure 26 K,L). Increased vessel density was observed in the vicinity of the tumor adjacent to the areas of leukocytic infiltration in the untreated group. Furthermore, significant increase in mitotic indices was observed in the untreated group as compared to $25OHD_3$ and $1,25(OH)_2D_3$ treated groups

In order to quantify and compare the difference in the mitotic index between groups ; we used the Ki-67 biomarker as proliferation index. Tumor sections stained with Ki-67 are shown in figure 27. Both $25OHD_3$ and $1,25(OH)_2D_3$ decreased the number of Ki-67 positive We quantified Ki-67 expression by the counting of 21 randomly selected microscopic fields. The proliferation index (Figure 27G) decreased from 50.2 ±8.6% in the untreated control group to 22.7 ± 11.3% (P = .002) and 29.7± 12.3% (P, <.005) in animals treated with $25OHD_3$ and $1,25(OH)_2D_3$ respectively.

## 3.5 Effects of $25OHD_3$ and $1,25(OH)_2D_3$ on Biomarkers of Tumor Progression

We next assessed the effects of $25OHD_3$ and $1,25(OH)_2D_3$ on tumor progression markers. Loss of estrogen receptor α (ERα) expression is associated with poor prognosis in human breast cancers (Figure 28, Figure 35 A)(320). In our model, the hyperplasic stage is associated with 30-40% loss of ERα positive cells, while the adenoma/MIN stage is associated with 50 to 80% loss of ERα positive cells. Progression into the early and late carcinoma stages is associated with 90% loss of ERα positive cells (Figure 20) (318). All tumors used for IHC were collected at sacrifice in this study. $25OHD_3$ treated animals had positive tumor staining for ERα of 80 to 90 % of cells examined (Figure 28 B,E, Figure 35 A) whereas $1,25(OH)_2D_3$ treated animals had ERα positive staining in 70-84% percent of cells (Figure28C,F).Untreated animals had only 10% ERα positive cells occupying most of the tumor sections examined (Figure28 A,D, Figure 35 A).

We next examined ERbB2/Neu, a constitutively actin EGF receptor expressed in all stages of tumor development but with markedly increased expression as tumors progress through the different stages (Figure 20)(321). The hyperplasic stage is characterized by light positive staining (10-25%) with a stepwise increase during the various stages of tumor progression. The untreated animal group expressed ERbB2/Neu in 85 % of all tumor cells analyzed with the most intense staining in the center of the tumor (Figure 29 A,D Figure 35 B).$25OHD_3$ and $1,25(OH)_2D_3$ treated animals had less than 10 percent of cells staining positive for ERbB2/Neu with markedly diminished intensity as compared to tumors from untreated animals (Figure 29 B,C,E,F, Figure 35B).

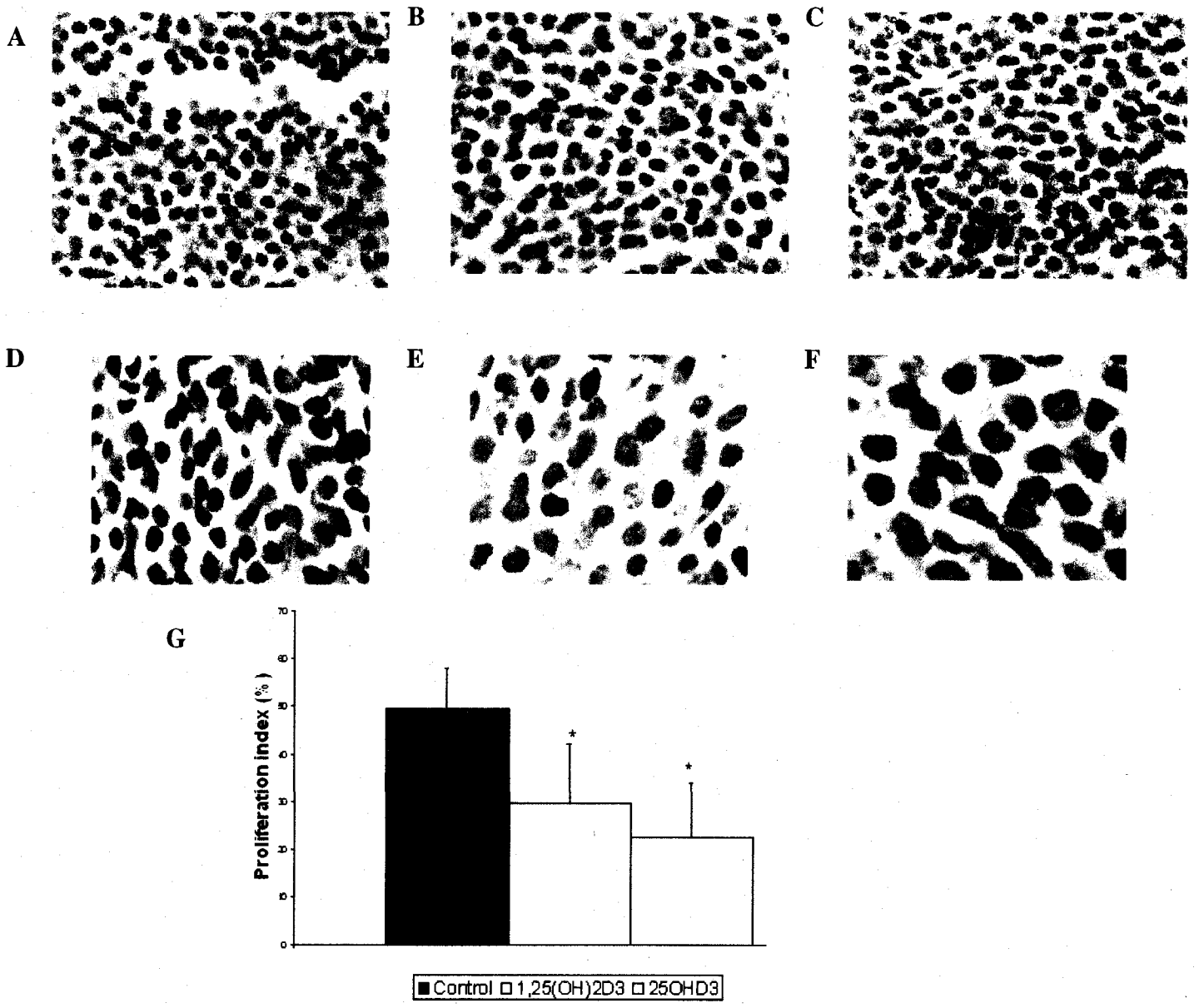



**Figure 27: Effect of $25OHD_3$ and $1,25(OH)_2D_3$ Compared to Vehicle on Ki-67 proliferation Marker in PyVMT Female Transgenic Mice.** Ki-67 staining of tissues sections: **(A,D)** representative micrographs of tumors from untreated animals;**(B,E)** representative micrographs of tumors from animals treated with $1,25(OH)_2D_3$ for 47 days;**(C,F)** representative micrographs of tumors from animals treated with $25OHD_3$ for 47 days.**(G)** Proliferation indices are calculated as the ratio of Ki-67 positive cells to the total number of cells in the selected microscopic fields. * indicates a significant difference from untreated animals ($P<0.005$) .Magnification of 200x for A-C and 400X for D-F. See text for further details

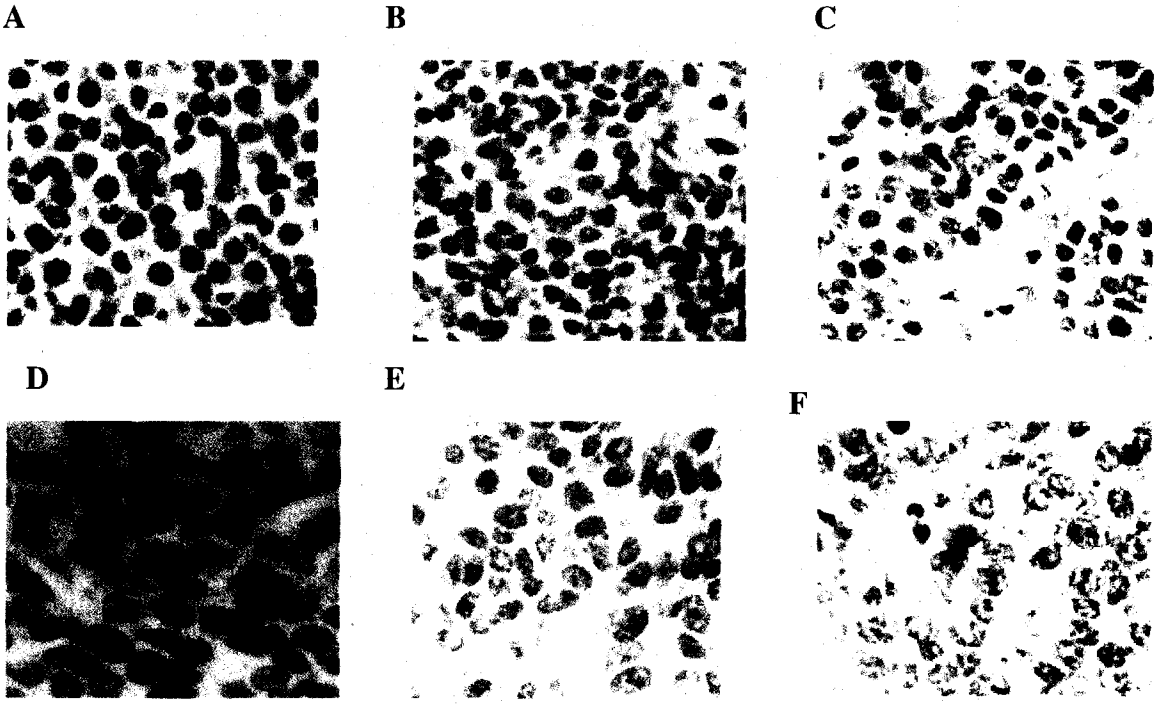



**Figure 28: ERα Expression in the PyMT Model**: ERα staining of tissues sections in breast tumors taken at sacrifice (12wks). **(A,D)** representative micrographs of tumors from untreated animals;**(B,E)** representative micrographs of tumors from animals treated with $1,25(OH)_2D_3$ for 47 days;**(C,F)** representative micrographs of tumors from animals treated with $25OHD_3$ for 47 days. Note the strong decrease of ERα in untreated control animals as compared to the $25OHD_3$ and $1,25(OH)_2D_3$ treated animals. Antibodies were purchased from Santa Cruz, CA .Magnification, 200x for A-C and 400X for D-F. See text for further details.

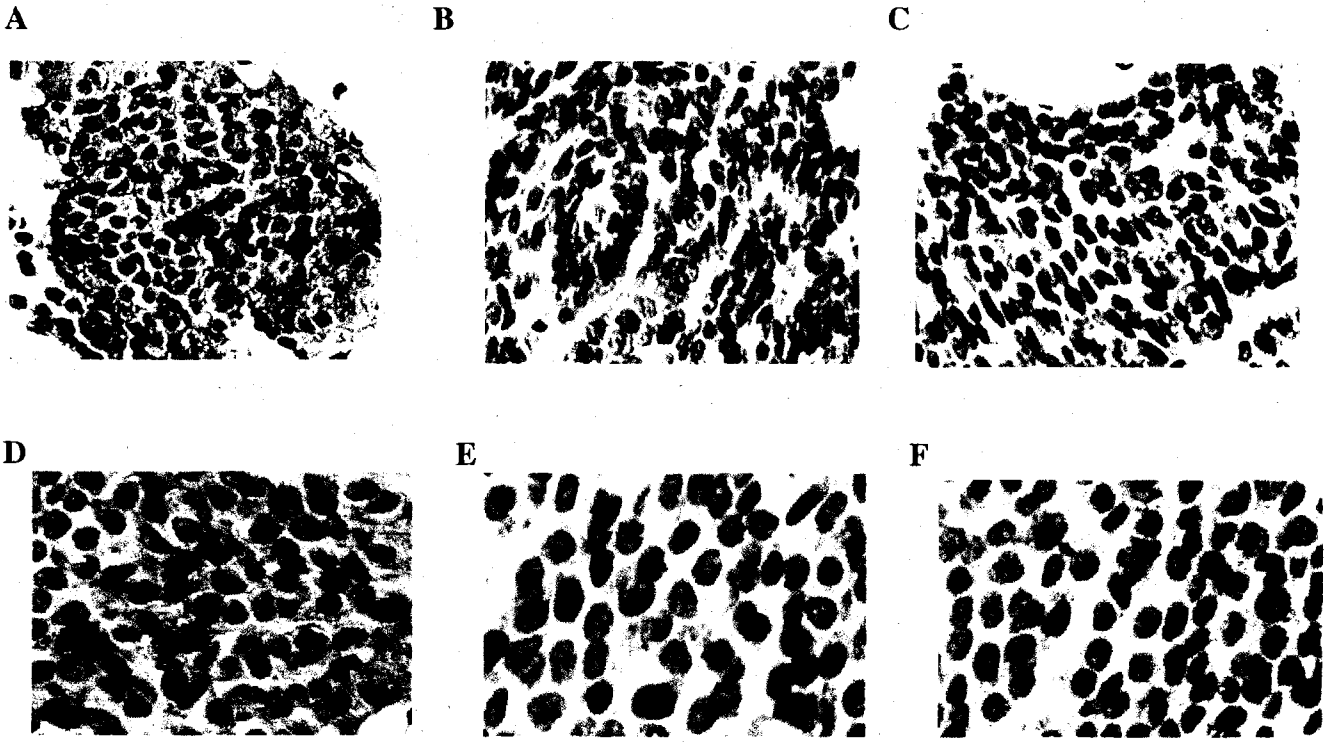



**Figure 29: Expression of ErbB2/Neu in PyMT Model:** ErbB2/Neu staining of tissues sections in breast tumors taken at sacrifice (12wks). **(A,D)** representative micrographs of tumors from untreated animals;**(B,E)** representative micrographs of tumors from animals treated with $1,25(OH)_2D_3$ for 47 days;**(C,F)** representative micrographs of tumors from animals treated with $25OHD_3$ for 47 days. Note the strong expression of ErbB2/Neu in vehicle-treated controls in comparison to the $1,25(OH)_2D_3$ and $25OHD_3$ treated animals. Antibodies were purchased from Santa Cruz, CA .Magnification, 200x for A-C and 400X for D-F. See text for further details.

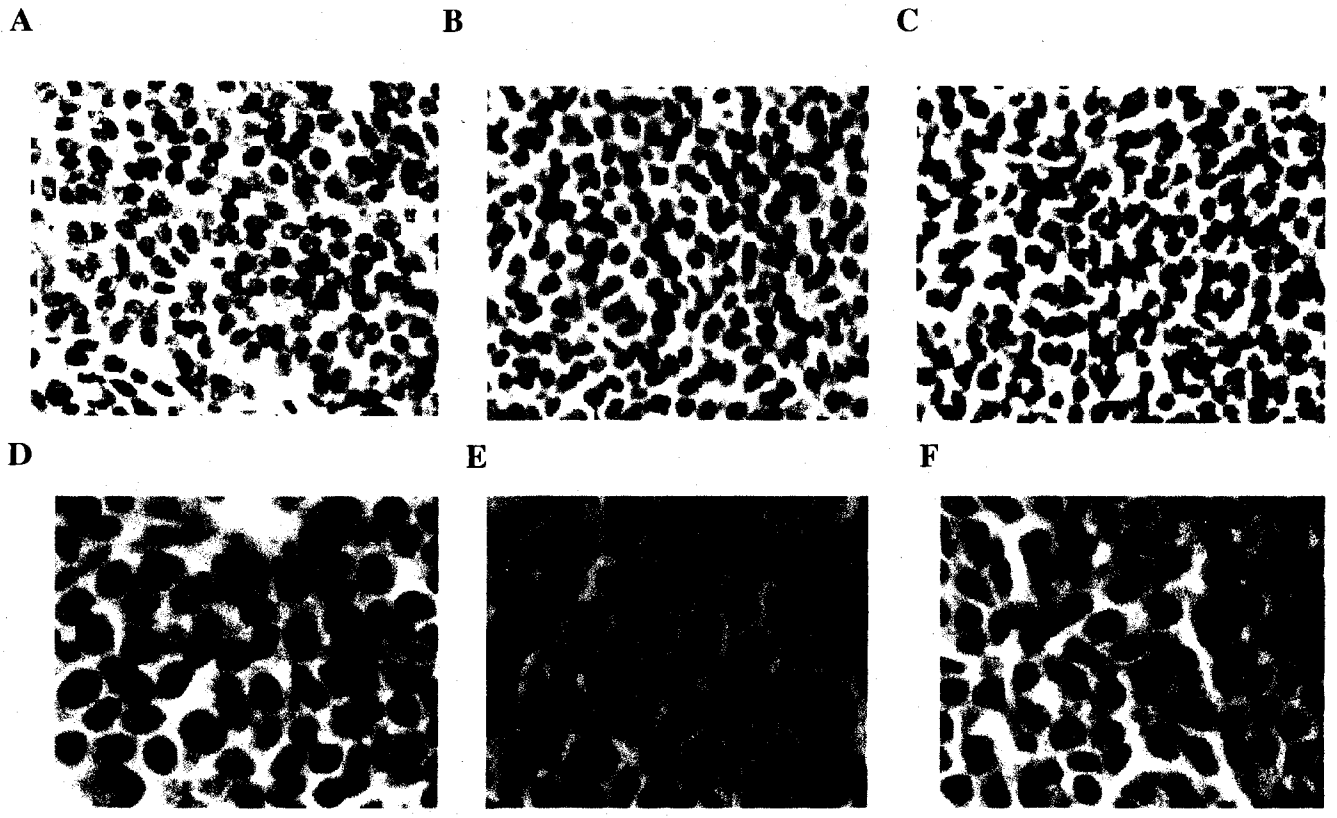



**Figure 30: Increased Expression of Cyclin D1 in Untreated Control Group.** Cyclin D1 staining of tissues sections in breast tumors taken at sacrifice (12wks) **(A,D)** representative micrographs of tumors from untreated animals;**(B,E)** representative micrographs of tumors from animals treated with $1,25(OH)_2D_3$ for 47 days;**(C,F)** representative micrographs of tumors from animals treated with $25OHD_3$ for 47 days. Note the strong inhibition expression of Cyclin D1 in $1,25(OH)_2D_3$ $25OHD_3$ treated groups in comparison to untreated control animals. Antibodies were purchased from Santa Cruz, CA .Magnification, 200x for A-C and 400X for D-F. see text for further details

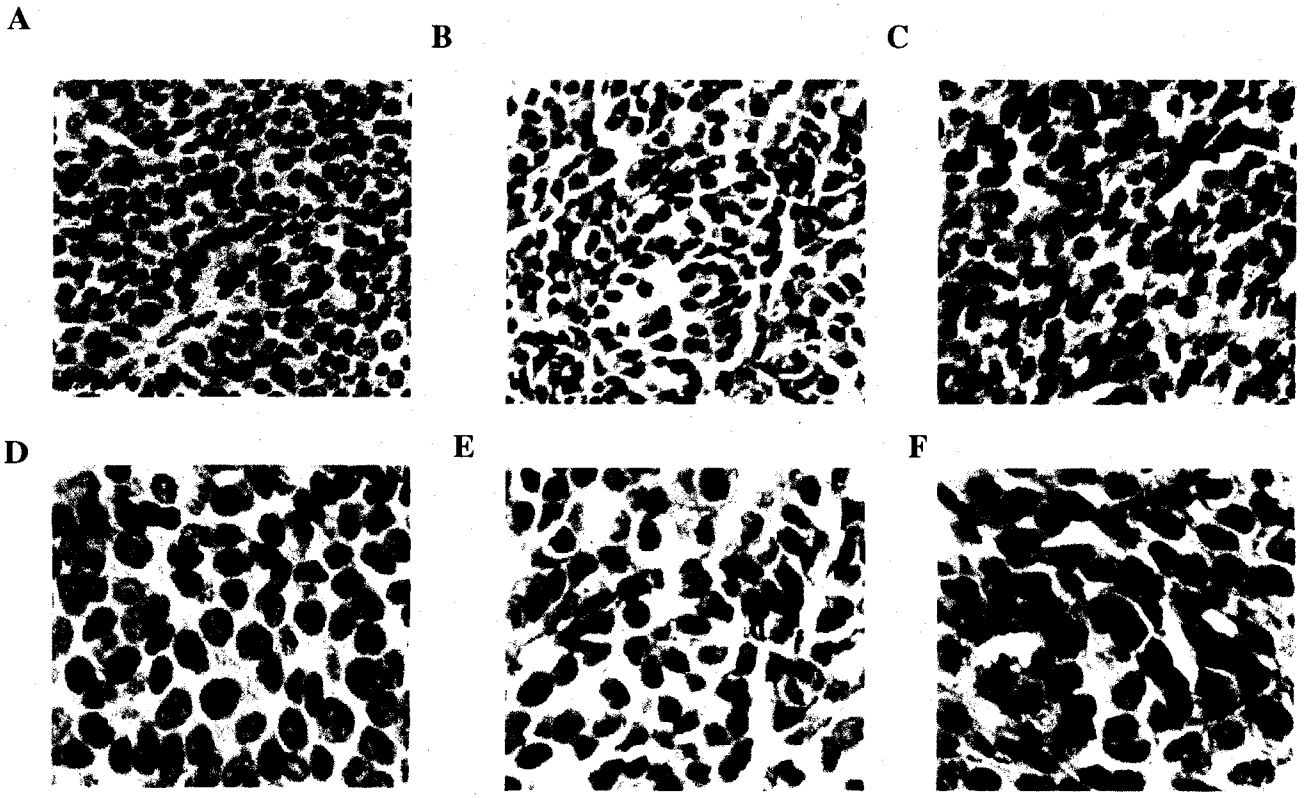



**Figure 31: Integrin β1 Expression in the PyMT Model**: Integrin β1 staining of tissues sections in breast tumors taken at sacrifice (12wks). **(A,D)** representative micrographs of tumors from untreated animals;**(B,E)** representative micrographs of tumors from animals treated with $1,25(OH)_2D_3$ for 47 days;**(C,F)** representative micrographs of tumors from animals treated with $25OHD_3$ for 47 days. Note the strong decrease of Integrin β1 expression in untreated control in comparison to the $25OHD_3$ and $1,25(OH)_2D_3$ treated animals. Antibodies were purchased from Santa Cruz, CA .Magnification, 200x for A-C and 400X for D-F. See text for further details.

We next assessed Cyclin D1, a regulator of G1 to S phase progression in many different cell types (274). Hyperplasia and MIN/adenoma stages were characterized by wide sparse distribution of cells staining positive for cyclin D1 of approximately 15-25% of cells. The late and early carcinoma stages were characterized by an even more dense distribution of areas that stained positive for cyclin D1 in 65-90% of cells. At the late carcinoma stage, a high density of cyclin D1 positive cells appears in clusters within the tumor (Figure 30 A,D). A similar pattern was observed in our untreated vehicle control mice with approximately 85% of cells staining for cyclin D1 (Figure 30 A,D, Figure 35 C). In contrast, tumors from $25OHD_3$ and $1,25(OH)_2D_3$ treated animals have less than 20% of cells staining for cyclin D1 in a random distribution and mostly located at the outer layer of the tumor acini(Figure 30 B,C,E,F, Figure 35 C).

Next we analyzed Integrin β1 expression, whose function is to maintain the orientation of the normal mammary epithelium and plays an important role in regulating cell proliferation, differentiation, and apoptosis of normal mammary epithelium (318,322,323). Changes in its expression are associated with tumorigenesis. Integrin β1 expression is associated with normal tissues and as tumor progression occurs Integrin β1 expression is lost. Early and late carcinoma is associated with little to no expression of Integrin β1 and loss of expression in the center of tumors, while the hyperplasic and min/Adenoma stage is associated with light staining of approximately 20-40% of cells.

In our experiments, untreated control mice were observed to have very light expression of Integrin β1 light with less than 10 % of tumor cells staining positive. (Figure 31 A,D Figure 35,D). $25OHD_3$ and $1,25(OH)_2D_3$ treated animals displayed strong positive staining in over 50% of the cells. Furthermore, tumors from these groups

displayed a high density of positively expressing cells in the center of the tumor as well as the outer ridge (Figure 31 B,C,D,E, Figure 35 D).

## 3.6 Effects of $25OHD_3$ & $1,25(OH)_2D_3$ on Lung Metastasis

PyVMT MMTV 634 transgenic mice mimic the metastatic potential of breast cancer with the development of pulmonary metastasis in late stage approximately between 12-13 weeks of age (296-300) (Figure 32). Macroscopic metastases are sometimes seen but microscopic metastases occur in 100% of animals by 12-13 weeks of age. Representative histological lung sections of $25OHD_3$, $1,25(OH)_2D_3$ and vehicle treated animals at time of sacrifice are illustrated in figure 32A-H. As expected, lung sections from untreated mice revealed metastatic tumor cells in 100% of animals sacrificed at 12-13 weeks which filled a substantial amount of lung space (Figure 32A,B). In contrast, the metastatic number and area of metastatic foci in the lung sections of $25OHD_3$ and $1,25(OH)_2D_3$ mice were significantly decreased (Figure 32 C-F).

In animals treated with $25OHD_3$, the mean number of metastases was $3.42 \pm 1.22$ per animal corresponding to a reduction of 73.4 % ($P<.002$) (Figure 33A). In animals treated with $1,25(OH)_2D_3$ the mean number of metastases was $5.14 \pm 1.67$ per animal corresponding to a reduction of 60% ($P<.05$)(Figure 33A) in comparison with $12.9 \pm 5.4$ of the control. The percentage of the lung area with metastases was 25% of control in the $25OHD_3$ ($P<.002$) (Figure 33B 32C,D) and 35% in the $1,25(OH)_2D_3$ treated group ($P<.05$) (Figure33B, Figure 33 E,F). Figures 32 G, H represent normal lung tissue from FVB Female mice.

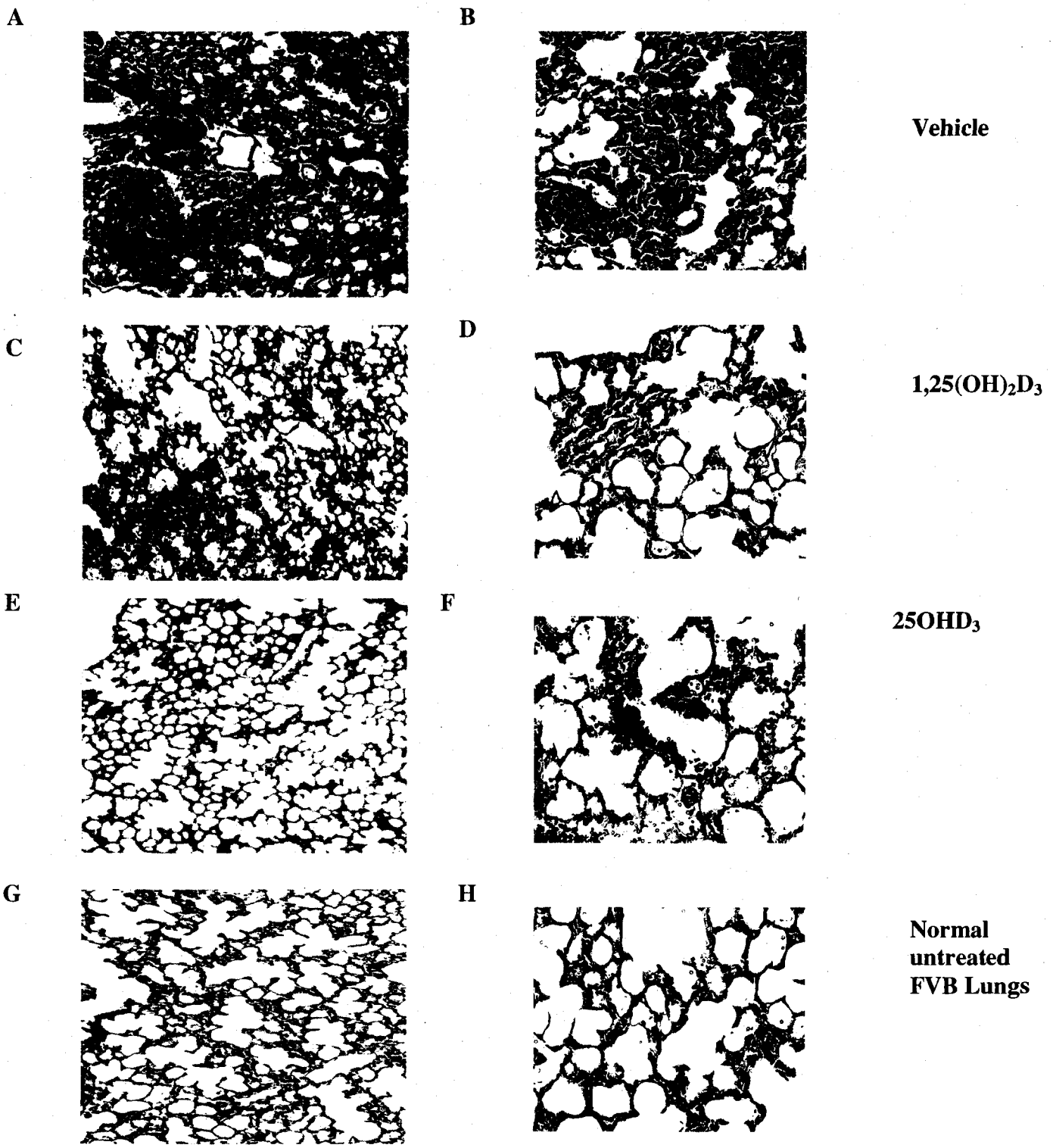
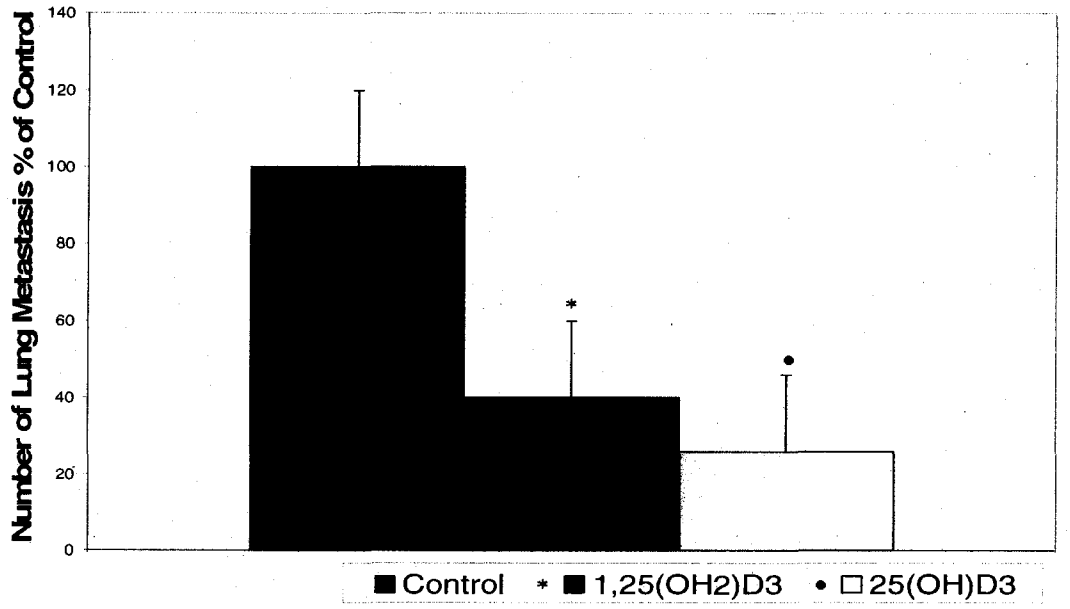



**Figure 32: Lung Histological Sections of $25OHD_3$ ,$1,25(OH)_2D_3$ and vehicle treated animals in the PyVMT Female Transgenic Mice.** Representative images of lung sections stained with H&E were captured through a computer attached to a microscope. **A** and **B** are untreated;**C** and **D** are treated with $1,25(OH)_2D_3$; **E** and **F** $25OHD_3$ treated PyVMT Female transgenic mice treated for 47 days . **G** and **H** are normal FVB lungs. Magnification 100x for A,C, E and G and 400X for B,D,F and H. See text for further details.

A

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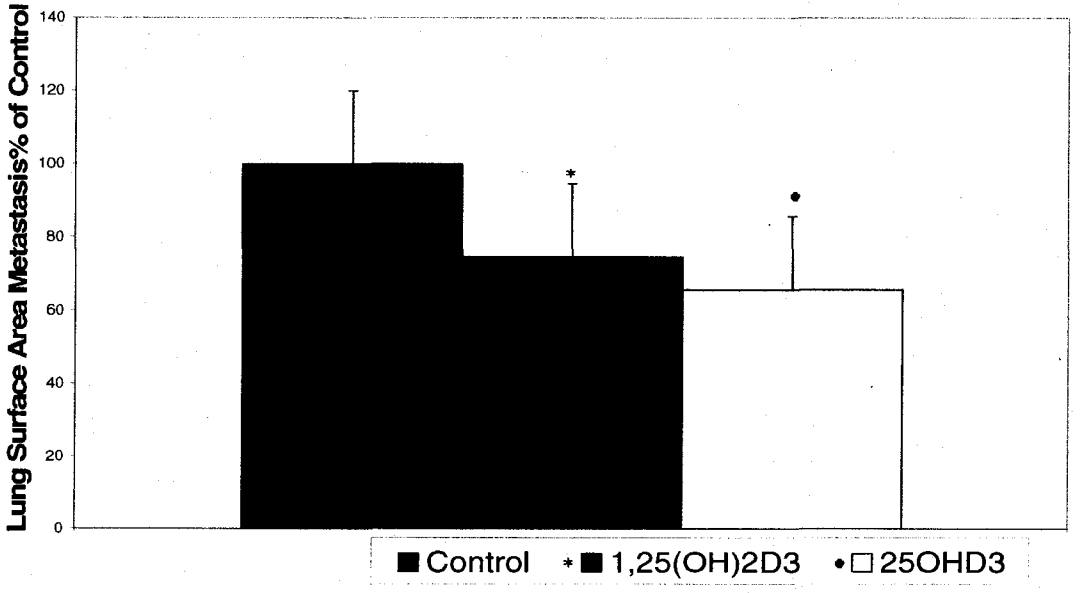


**Figure 33: Effects of $25OHD_3$ and $1,25(OH)_2D_3$ on the Number and Surface Area of metastatic Lung Foci in the PyVMT MMTV #634 Female Transgenic Mice.** Number and surface areas were scored in a genotype-blinded fashion under low power using a Nikon SMZ-1500 stereomicroscope. The total tissue area (metastases and normal lung tissue) was calculated using software provided by the Center for Bone & Periodontal Research (Montreal Quebec).After this had been completed for all slides each slide was then examined again and areas of metastatic tissue were calculated. The total area of metastatic tissue were then subtracted from the total surface area and compared between groups. The percentage of metastatic surface area was then calculated for the $25OHD_3$,$1,25(OH)_2D_3$ and vehicle treated animals. These percentages were then expressed as a percent of control of vehicle treated animals. **(A)** The number of metastatic lesions expressed as a percent of control of vehicle treated animals. **(B)** The metastatic surface area represented as a percent of control of vehicle treated animals.

## 3.7 Effects of $25OHD_3$ and 1, $25(OH)_2D_3$ on Plasma Calcium Concentrations

Only tumor bearing animals that received $1,25(OH)_2D_3$ infusion showed a significant ($P<.05$) increase in plasma calcium concentrations as compared to vehicle-treated control group. Animals treated with $25OHD_3$ showed no significant increases in calcium concentrations (Figure 34,Table 4). $1,25(OH)_2D_3$ is known for its hypercalcemic effects (17,50,56) and accordingly our $1,25(OH)_2D_3$ treated group at sacrifice showed increased mean calcium concentrations of 3.19±.91 mmol/L as compared to 2.38±.16mmol/L in vehicle treated animals (Figure 34). In contrast, $25OHD_3$ treated animals remained within the normal range of 2.39±.13mmmol/L (Table 4). Serum albumin is an indicator of nutritional status and hepatic function (324). It also binds calcium and it is a concomitant measure used for correction. During tumor progression, the mean serum albumin levels of vehicle treated mice decreased by 14.0±2%, $1,25(OH)_2D_3$ treated animals by 18.3±.1.9% and $25OHD_3$ by 12.3.8±.1.75% . Creatinine was measured as an indicator of kidney function since elevated levels of creatinine are indicative of kidney impairment (326). The mean serum creatinine levels of vehicle-treated mice increased by 31.0±1.9%, $1,25(OH)_2D_3$ treated mice had an increase of 40.0±.9.8% and $25OHD_3$ treated mice had an increase of 38.2.±.7.6.% from pretreatment values (Table 4). Serum alkaline phosphatase was also measured as an indicator of liver and bone status (326). At sacrifice, the vehicle treated animals had the highest mean levels of alkaline phosphatase of 90.75±31.6U/L followed by the $1,25(OH)_2D_3$ treated group of 72.4±19.2U/L and the $25OHD_3$ treated group of 69.85±15.8U/L .The tissue origin of the alkaline phosphatase is not known and analyses of isoenzymes are underway

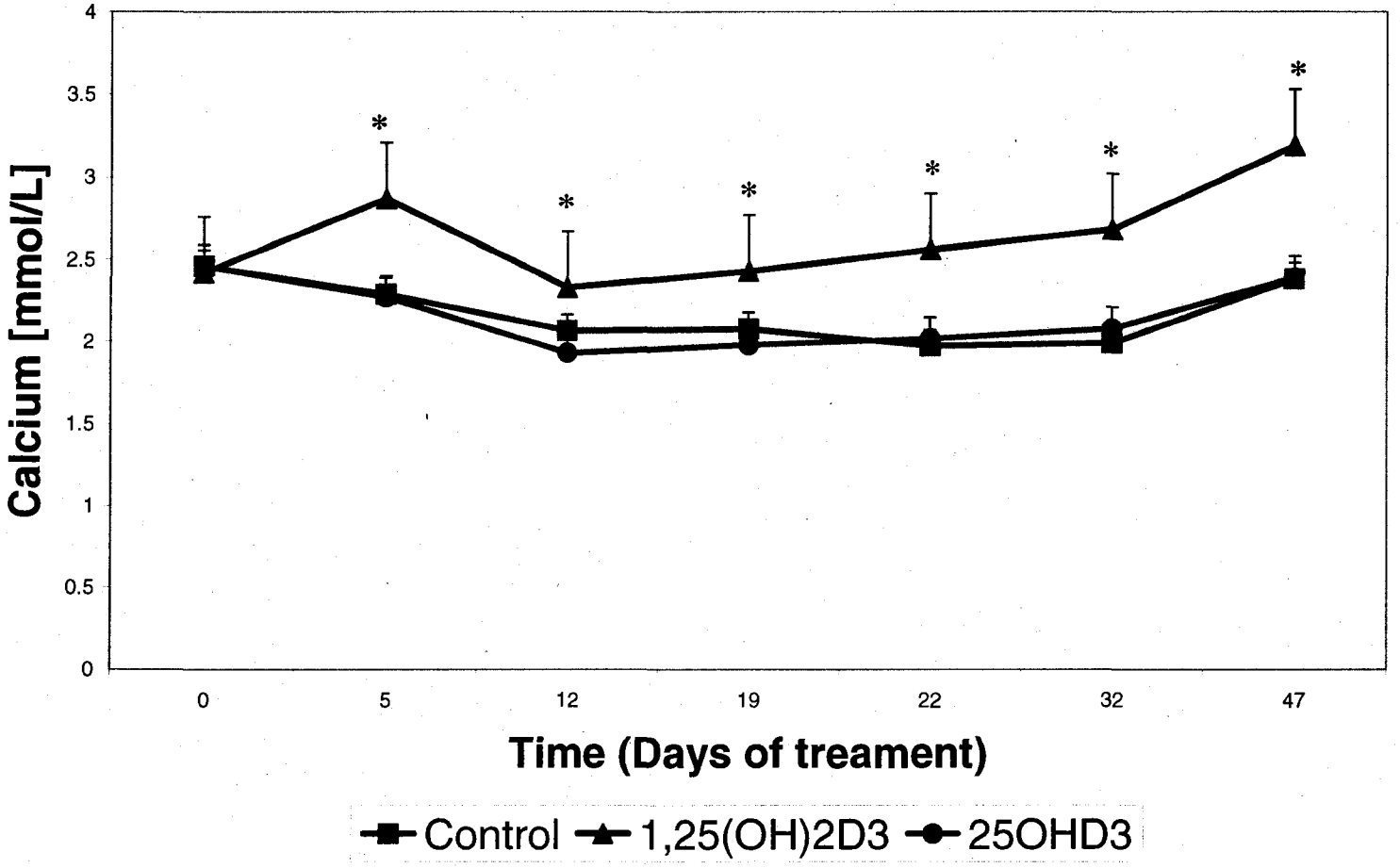


**Figure 34: Serial Measurements of Blood Calcium Concentrations**. Mice were bled once a week, and their plasma calcium determined as described in "Materials and Methods." Results represent the means ± SE of calcium concentrations of mice in each group; bars, SE

**Table 4: Plasma Concentrations of Biochemical Parameters in 25OHD$_3$, 1,25(OH)$_2$D$_3$ and Vehicle Treated Animals at Sacrifice**

Serum

| | | Total Calcium (mmol/L) | Albumin (g/L) | Phosphorus (mmol/L) | Creatinine (mmol/L) | Alkaline Phosphatase (U/L) |
|---|---|---|---|---|---|---|
| Untreated | (n=10) | 2.38 ± .16 | 26.0 ± 2.00 | 4.50 ± .58 | 26.50 ± 1.96 | 90.75 ± 31.6 |
| 1,25(OH)$_2$D$_3$ | (n=9) | 3.19 ± .91 * | 27.3 ± 1.96 | 4.37 ± 1.18 | 30.46 ± 9.18 | 72.40 ± 19.2 * |
| 25OHD$_3$ | (n=12) | 2.39 ± .13 | 26.28 ± 1.79 | 4.23 ± .67 | 25.08 ± 7.67 | 69.85 ± 15.8 + |

Results are mean ± S.E.M

P value of <0.05 was considered significant *

* Indicates a significant difference between the serum ion concentrations of the untreated group in comparison to that of the 1,25(OH)$_2$D$_3$ treated group

+ Indicates a significant difference between the serum ion concentrations of the untreated group in comparison to that of the 25OHD$_3$ treated group

Plasma Concentrations of Biochemical Parameters were taken at Sacrafce at 12 weeks of age

**Table 5: Tissue Concentrations of 25OHD$_3$ and 1,25(OH)$_2$D$_3$ from Breast Tumors and Kidneys Extracts**

| | | Tumor Tissues | | Kidneys | |
|---|---|---|---|---|---|
| | | ng/g 25OHD$_3$ | pg/g 1,25(OH)$_2$D$_3$ | ng/g 25OHD$_3$ | pg/g 1,25(OH)$_2$D$_3$ |
| Untreated | (n = 10) | 95.0 ±15.47 | 182.0 ± 10.5 | 261.0 ± 12.48 | 389.0 ± 26.9 |
| 1,25(OH)$_2$D$_3$ | (n = 9) | 69.0 ±6.09 | 326.0 ± 21.8 * | 207.0 ± 15.8 | 372.0 ± 33.0 |
| 25OHD$_3$ | (n = 12) | 650.0 ±35.72 + | 390.0 ± 16.7 • | 583.0 ± 28.61 + | 390.0 ± 25.5 |

[a] Results are mean ± S.E.M.

A P value of <0.05 was considered significant

+ Indicates a significant difference in 25OHD$_3$ concentrations of animals treated with 25OHD$_3$ as compared to animals of the untreated group.

• Indicates a significant difference in 1,25OH$_2$D$_3$ concentrations of animals treated with 25OHD$_3$ as compared to animals of the untreated group.

* Indicates a significant difference in 1,25OH$_2$D$_3$ concentrations of animals treated with 1,25OH$_2$D$_3$ as compared to animals of the untreated group.

to determine its origin. Finally we measured phosphorus concentrations which increased in comparison to their initial values but was not significant over time in all three groups (Table 4). The increase in phosphorus concentrations may likely represents the effects of tumor burden and metastatic spread in all treated groups.

## 3.8 Measurement of Vitamin D Metabolites in Tissue Samples

In order to shed light on the mechanism of $25OHD_3$ induced tumor inhibition, we next measured $25OHD_3$ and $1,25(OH)_2D_3$ in tumor tissues at sacrifice and in kidney tissues as controls (Table 5). Blood levels were also measured at sacrifice. In mice treated with $1,25(OH)_2D_3$ a 79.0 % increase in $1,25(OH)_2D_3$ concentrations in tumor tissues was observed as compared to vehicle-treated animals. $1,25(OH)_2D_3$ concentrations measured in the kidney extracts, remained unchanged as compared to the untreated group (Table5). Quantification of tumoral $25OHD_3$ concentrations in mice treated with $25OHD_3$ increased six fold as compared to the untreated group. Furthermore, quantification of $1,25(OH)_2D_3$ concentrations in the $25OHD_3$ treated group indicated an increase of 114.3% in tumoral $1,25(OH)_2D_3$ as compared to untreated group. In contrast, kidney extract concentrations of $1,25(OH)_2D_3$ did not increase (Table 5).Thus, the increased levels of $1,25(OH)_2D_3$ in mammary tumor tissues reflects the lack of 1αOHase regulation in mammary tumor tissues in comparison to the strict regulation that is seen in the proximal tubule cells of the kidneys.

## 3.9 Effects of $25OHD_3$ and $1,25(OH)_2D_3$ on Tumoral 1α Hydroxylase Expression.

Tumors from all groups were collected at sacrifice and 1αOHase levels were quantified (Figure 35 E). As shown in figure 35 E, expression of 1αOHase /CYP27B1 did not decrease in either $1,25(OH)_2D_3$ or $25OHD_3$ treated groups as compared to the untreated control group. In the kidneys, the $1,25(OH)_2D_3$ product of $25OHD_3$ conversion by 1αOHase inhibits its own production by a feedback mechanism(50,54). Our data therefore indicates that 1αOHase in tumor tissues is not subjected to this feedback loop and may therefore be beneficial in tumor cells attempting to decrease cell proliferation in an autocrine manner.

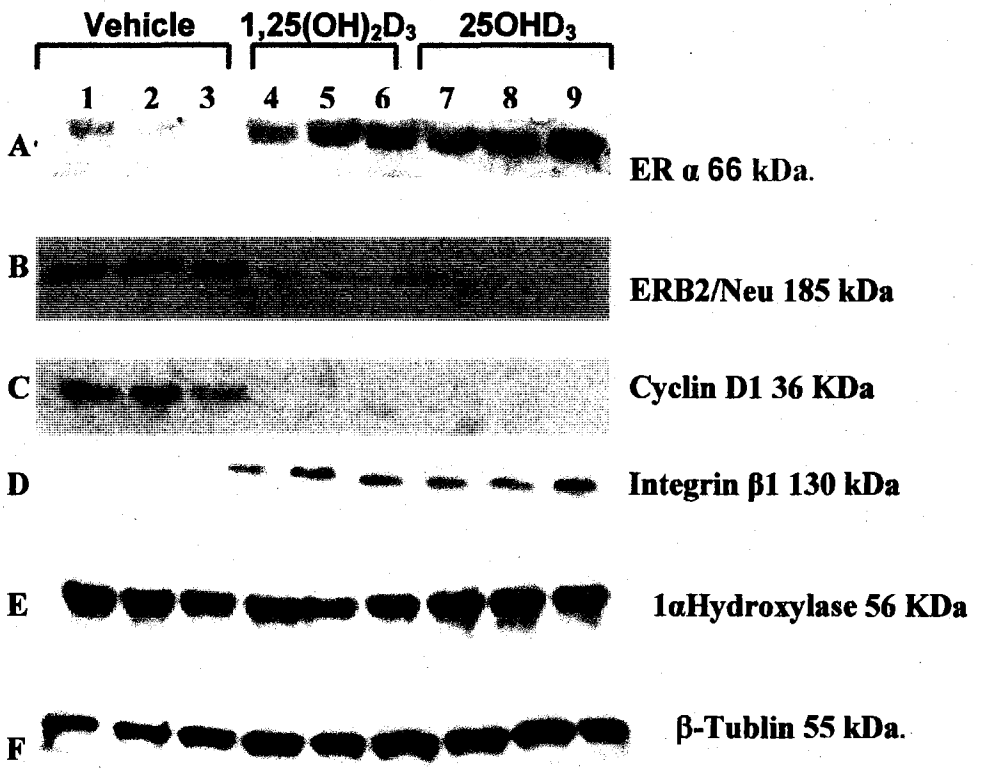


**Figure 35.Western blot analysis of Tumor Cell Extracts**. Tumor Breast extracts taken at sacrifice (12wks) were analyzed by western blotting for protein expression levels of **(A)** ERα, **(B)** ERB2/Neu, **(C)** Cyclin D1, **(D)** Integrin β1, **(E)**1αhydroxylase and **(F)**β-Tubulin. Lanes 1-3 are tumor extracts from the vehicle-treated control group, Lanes4-6 are tumor extracts from the $1,25(OH)_2D_3$ treated animals and lanes 6-9 are from $25OHD_3$ treated animals.

# Chapter 4: Discussion

## 4.1 The Importance of 1,25-dihydroxyvitamn $D_3$

In the present study, we have shown that $25OHD_3$ significantly suppress the growth of mammary carcinomas *in vivo* in the transgenic Mice model of the Polyoma middle T antigen mouse mammary tumor virus strain 634 mice. VDRs are expressed in more than 80% of human breast cancers (328,329) and consequently $1,25(OH)_2D_3$ has been identified as a potential agent in the treatment of breast cancer (327,330,333-340).However, its strong calcemic activity prevents its usefulness in this disease (326). Analogues of $1,25(OH)_2D_3$ have been developed to circumvent this problem hoping that they will maintain the anti-proliferative and differentiation attributes of 1,25-dihydroxyvitamn $D_3$ while keeping normal physiological calcium levels (11,333-338). $25OHD_3$ is $1,25(OH)_2D_3$ precursor that is synthesized in the liver and is inactive per se (50,51). In view of the fact that the 1αOHase enzyme which converts $25OHD_3$ into $1,25(OH)_2D_3$ is expressed in breast tumor cells; its use may be advantageous to avoid side effects even at very high concentrations such as the one used in our study. This approach is particularly important because of the potential to treat breast cancer patients. The production of sufficient amounts of 1,25-dihydroxyvitamn $D_3$ from $25OHD_3$ in breast cancer cells by CYP27B1 without inhibition by the $1,25(OH)_2D_3$ negative feedback loop shows potential to achieve sufficient local concentrations in the tumor that may act to inhibit tumor progression through an autocrine affect without the hypercalcemic side effects of $1,25(OH)_2D_3$.

## 4.2 Modulation of Histopathological Indices of Tumor Progression and Biomarkers

We demonstrated that $25OHD_3$ and 1,25-dihydroxyvitamn $D_3$ not only inhibited the tumor onset of the primary tumor but also inhibited the secondary tumor appearance as well. Total primary group tumor onset for the $1,25(OH)_2D_3$ and $25OHD_3$ treated groups were determined at 23 and 27 days post treatment as compared to 9 days post treatment in the vehicle treated control group. This is a delay of 48 and 57% respectively. Moreover, the total secondary group tumor onset for the $1,25(OH)_2D_3$ and $25OHD_3$ treated group was determined at 27 days post treatment as compared to 9 days post treatment in the vehicle treated control group which is a delay of secondary tumor onset of 57% percent in comparison to the tumor onset observed in the control group.

$25OHD_3$ and $1,25(OH)_2D_3$ treated animals at sacrifice were observed to have an overall reduction of tumor volume of 58±2.1% and 45±2.5 % respectively. Additionally, at sacrifice the untreated vehicle control group average tumor volume was 1.8 times the volume of the $1,25(OH)_2D_3$ and 2.4 times the volume of the $25OHD_3$ treated groups. On days 6 and 9 the standard deviation appears to be quite elevate in relation to tumor size which can be explained by the fact that each member of each group experienced a different rate of tumor on set and growth with in the group itself which is represented by the large standard deviation at these time points. However, once all mouse groups experienced tumor onset and growth, the standard deviation between each member of each group began to decrease and plateau as seen by latter time intervals The Kaplan-Meier analysis of tumor onset supports this reasoning.

25OHD$_3$ and 1,25(OH)$_2$D$_3$ treated groups had significantly reduce tumor burden in the mammary epithelium. Additionally, the 1,25(OH)$_2$D$_3$ and 25OHD$_3$ treated groups upon palpitation and sacrifice were observed to have mammary tumors restricted to mostly the upper mammary glands while the untreated vehicle control group had rampant, widespread mammary tumor growth and proliferation in all of the 10 mouse mammary glands. The total number of tumors was tabulated upon sacrifice per mouse per group and illustrated an overall significant mean reduction of tumor number in the 1,25(OH)$_2$D$_3$ and 25OHD$_3$ group as compared to the untreated vehicle control group. A reduction of 66.6% in tumor number was observed for the 25OHD$_3$ treated group. The mean weight of mice in all groups progressively increased with the increased tumor volume and number. The untreated vehicle control group exhibited an overall increase in total body weight of approximately 56 ±0.7% (Figure 24B) as compared to 26.8±0.8 % ($P<.002$) in the 25OHD$_3$ and 35.3±.3% ($P<.05$) in the 1,25(OH)$_2$D$_3$ treated group (Figure 24 B).Body weight is usually an indicator of the overall state of health of an animal and decreases in body weight are usually associated with animals that are becoming progressively sicker. However, all groups showed a progressive mean increase in body weight which is due to the rapid growth of tumors in relation to the overall body size and the duration of the experiment. At day 6, the vehicle control group experienced an increase in body weight being which corresponds with the increase tumor volume as seen in figure 24A. Moreover, mice appeared to gain weight even though they were becoming sicker because the gain in weight is not due to nutritional gain but due to rapid increase in tumor size and volume.

Mice at 12 weeks of age were sacrificed and the lungs exposed by thoracic and tracheal dissection. Nine lung lobes were separated in order to visualize all surfaces of each lobe. $25OHD_3$ and $1,25(OH)_2D_3$ were observed to have a strong inhibition of lung metastasis with a reduction of 73.39 %($P<.002$) and 60% ($P<.05$) in the $25OHD_3$ and $1,25(OH)_2D_3$ treated group respectively as compared to vehicle. Furthermore, the metastasis surface area was reduced by 75 % and 65 % of control in the $25OHD_3$ and $1,25(OH)_2D_3$ treated groups respectively.

Histological examination of tumors from the different groups clearly demonstrated $25OHD_3$ and $1,25(OH)_2D_3$ inhibitory potential. Histological examination of $25OHD_3$ and $1,25(OH)_2D_3$ treated tumors showed a lower stage of tumor progression (premalignant -MIN/Adenoma) at comparable tumor burden whereas tumors from untreated vehicle control were all characterized by invasive late carcinoma at sacrifice . Untreated tumors exhibited loss of acini structure and the loss of well defined boundaries. Furthermore, malignant tumor cells lacked specific defined morphology and exhibited a wide range of variability in cellular and nuclear size with pleiomorphic variation in nuclear morphology, size and shape. In contrast, tumors from animals treated with $25OHD_3$ or $1,25(OH)_2D_3$ had well defined densely packed lobules with ornate epithelial proliferation still confined by a basement membrane and connective tissue with minimal cytological atypia. Additionally, tumors from $25OHD_3$ and $1,25(OH)_2D_3$ treated animals had decreased vascularization and lymphocyte infiltration in comparison to the vehicle-treated control group.

This was further confirmed by a mechanistic approach involving Immunhistochemistry and western blotting utilizing a series of biomarkers associated with poor prognosis in human breast cancer that are mimicked in the same manner as in our model. Loss of ERα is associated with poor prognosis (320). Consequently, the

vehicle control group exhibited a drastically marked reduction in the amount of ERα positively expressing cells of approximately 90% while $25OHD_3$ and $1,25(OH)_2D_3$ treated animals had positive tumor staining for ERα of 80 to 90 % and 70-84% of cells examined respectively (Figure 28 BC, E F, Figure 35 A). ERB2/Neu is a constitutively actin EGF receptor expressed in all stages of tumor development but with markedly increased expression as tumors progress through the different stages and its expression is associated with poor prognosis (321). $25OHD_3$ and $1,25(OH)_2D_3$ treated animals had less than 10 percent of cells staining positive for ERbB2/Neu with markedly diminished intensity as compared to tumors from untreated animals which exhibited a positive staining of more than 85 %. Cyclin D1, a regulator of G1 to S phase progression in many different cell types expression is associated with poor prognosis as increased expression is associated with increased tumor progression (274). Levels of expression were markedly elevated in the vehicle-treated control group with more than 85 % of cells expressing the cell cycle regulator, while levels of expression where markedly reduced in the $25OHD_3$ and $1,25(OH)_2D_3$ treated groups of the magnitude of less than 20% . Integrin β1 expression, whose function is to maintain the orientation of the normal mammary epithelium, was severely diminished in the untreated vehicle control. As mentioned before the overall structural morphology of tumors originating from the untreated control group was greatly altered revealing the lack of clear, specific defined morphology and exhibited a wide range of variability in cellular and nuclear size with pleiomorphic variation which can be attributed to the loss of Integrin β1.However the $25OHD_3$ and $1,25(OH)_2D_3$ treated groups exhibited an intense staining of the protein which corresponds with the more defined structural characteristics. The proliferation index utilizing Ki-67 is routinely used to characterize the histopathological grade of neoplasms (238).Ki-67 staining decreased from 50.2 ±8.6% in the vehicle treated

controls to 29.7± 12.3% (P, <.005) and 22.7 ± 11.3% (P = .002) in $1,25(OH)_2D_3$ and $25OHD_3$ groups respectively (Figure 20,21).

The $1,25(OH)_2D_3$ treated group experienced a progressive increase in their circulating concentration levels of calcium that resulted in a state of hypercalcemia within the first week of administration. Consequently, the $1,25(OH)_2D_3$ treated grouped had a 25 % increase in comparison to $25OHD_3$ and vehicle-treated control group. A marked increase in alkaline phosphatase and phosphorus levels were seen in the vehicle-control group at sacrifice in comparison to their initial values which may be associated with malnourishment, increased tumor burden, and decreased liver function. $25OHD_3$ treated animals did also experience this marked increase in alkaline phosphatase and phosphorus levels in comparison to its initial values. However animals treated with $1,25(OH)_2D_3$ experienced increases in calcium, creatinine, phosphorus and alkaline phosphatase which may be indicative of overall degradation of overall health which are due to the hypercalcemic effects of $1,25(OH)_2D_3$ which ultimately lead to malnourishment, decrease renal and hepatic function. The increased phosphorus values may be indicative of this possibility and can potentially rule out that increased circulating concentration levels of $1,25(OH)_2D_3$ have acted to increase phosphorus absorption for the untreated control group and the $25OHD_3$ treated animals also experienced similar increases in phosphorus serum concentrations as compared to their initial values as well. Analysis of isoenezymes is underway to better understand the effect on hepatic function.

The data indicates a strong correlation with the histological characteristics and stage classification of tumors originating from these different groups. Furthermore it demonstrates the use of $25OHD_3$ and $1,25(OH)_2D_3$ as a tumor suppressor agent that

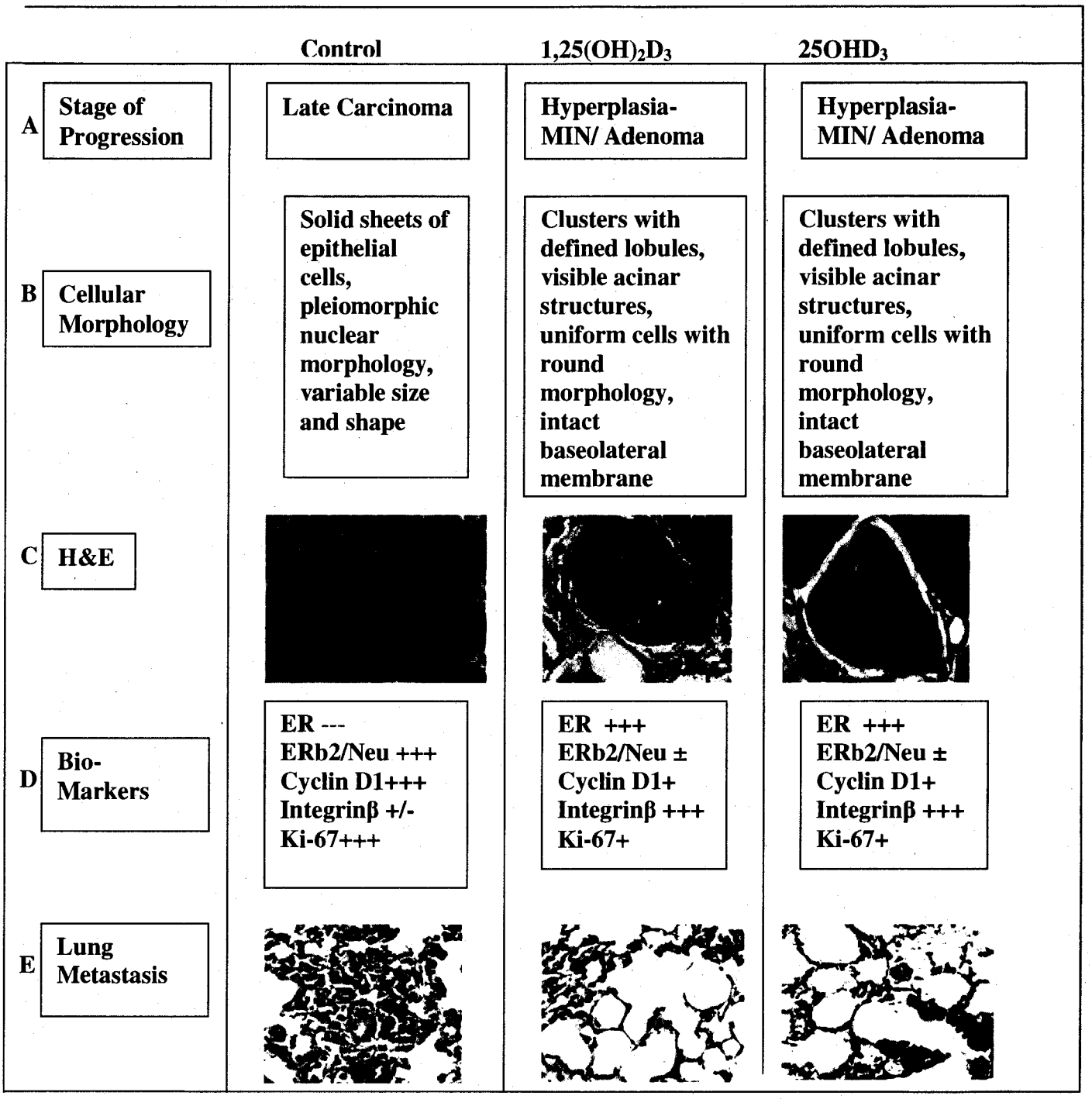


**Figure 36.** Summary of Tumor Progression and Biomarker Expression in **25OHD$_3$, 1,25(OH)$_2$D$_3$ and vehicle Treated Animals in the PyVMT Female Transgenic** Mouse Model of Breast Cancer. (A) Displays the overall classification of group tumor stage. (B) The cellular morphology panel schematically illustrates changes in the cytology of the cells as well as the integrity of the basement membrane and the presence or absence of myoepithelial and focal inflammation. (C) H&E panel displays the corresponding histology at sacrifice. (D) Changes in biomarkers expression between the 25OHD$_3$,1,25(OH)$_2$D$_3$ and vehicle treated animals at sacrifice.(E) H&E panel displays the extent of lung metastasis between 25OHD$_3$,1,25(OH)$_2$D$_3$ and vehicle treated animals corresponding histology at sacrifice.

results in a significant reduction in tumor number ,size and number of lung metastasis. However it also demonstrates that $25OHD_3$ is capable of inducing all of vitamin Ds beneficial characteristics without inducing a state of hypercalcemia Figure 36 summarizes the above mentioned results.

## 4.3 Significance of 1αOHase Tumoral Expression

To better understand the mechanism of $25OHD_3$ and $1,25(OH)_2D_3$ inhibition on mammary carcinoma progression, we analyzed tumoral 1αOHase (CYP27B1) expression at different stages of tumor progression. Normal breast tissue and tumor tissues at the different stages of tumor progression were also analyzed. Tumors extracted at the hyperplasia stage (premalignant lesion) (5-7weeks of age), adenoma/mammary intraepithelial neoplasia (8-9 weeks) and late carcinoma stage (10-13 weeks of age) did not show any difference in 1αOHase expression. Furthermore, quantitative analysis by western blotting of tumoral 1αOHase at sacrifice in all groups did not show altered expression between the basal physiological levels and those found in the tumor tissues at any of the different stages of tumor progression. Furthermore, we clearly demonstrated and eliminated the possibility that the affects we were observing, being, the significant reduction in tumor size, number, the number of lung metastasis, reduction in negative and increase in positive prognostic biomarkers is due to the increased induction of local 1αhydroxylase. For it is evident that upon viewing figure 22 A-D and Figure 35 E that the local mammary expression of local 1αhydroxylase does not change during tumor progression nor upon administration of $25OHD_3$ or $1,25(OH)_2D_3$.

We further analyzed 1αOHase expression in vitamin D treated animals (12pM/24h) who experienced a 79.0% increase in mammary tumoral circulating vitamin $D_3$ concentration levels as compared to the untreated vehicle control. This demonstrated

the lack of negative regulation of 1αOHase in response to increased local 1,25-dihydroxyvitamn $D_3$ concentrations in the mammary tumoral tissue samples. Moreover, we also analyzed 1αOHase expression in $25OHD_3$ treated animals (2000 pM/24h) who experienced an increase of 114.3% in tumoral $1,25(OH)_2D_3$ concentrations as compared to the untreated group but still did not experience the negative feedback inhibition that would be observed at the level of the renal system. Additionally,1αOHase expression did not change between any of the three groups as seen in Figure 35E.Consequently, we measured the serum concentration levels of circulating $25OHD_3$ and $1,25(OH)_2D_3$ at the renal level of $25OHD_3$ and $1,25(OH)_2D_3$ treated mice. We observed the rapid response of the negative feedback loop which maintained the circulating level of 1,25dihydroxyvitamin $D_3$ with in all three groups with in the same concentration range. $1,25(OH)_2D_3$ at the level of renal system were maintained within the normal circulating parameters, however $25OHD_3$ concentration levels increased 123.4% which is expected as we administered 2000 pM/24h. Additionally, $25OHD_3$ mammary tumoral concentration levels were increased by 6 fold in comparison to untreated and $1,25(OH)_2D_3$ animals which resulted in the 114.3% increase in tumoral $1,25(OH)_2D_3$ concentration levels. The degree of tumor inhibition that has been detected between the $25OHD_3$ and $1,25(OH)_2D_3$ groups is explained by the difference in tumoral circulating concentration levels of $1,25(OH)_2D_3$ . The extent of the inhibition has been relatively similar between the two groups and may be explained by the percent difference between them of only 35%.However the question may arise that perhaps the effects that we are observing may be due to a direct effect of $25OHD_3$ on the vitamin D receptor independent of the hydroxylases conversion of $25OHD_3$ to $1,25(OH)_2D_3$ or an autocrine effect. However, the necessary concentration of $25OHD_3$ that is required to competitively displace $1,25(OH)_2D_3$ from the VDR and to activate the genomic machinery that

potentates the anti proliferative and inhibitory characteristics of vitamin D are of the magnitude of approximately 1000nM (938nm specifically) which is a concentration that is significantly higher than concentration that was used in this experiment (2000pM/24h) (363). Thus, we have circumstantially demonstrated that due to the lack of negative feedback inhibition within the mammary tumoral tissue, excessive concentrations of $1,25(OH)_2D_3$ are produced via conversion from its metabolite $25OHD_3$ which then causes the inhibition of tumor progression of mammary carcinomas through an autocrine mechanism. Though this does not clearly demonstrate that $25OHD_3$ that is administered to the mice is actually converted to $1,25(OH)_2D_3$ at the tumoral level, it is clear that the subcutaneous administration of $25OHD_3$ is resulting in an increase in tumoral $1,25(OH)_2D_3$ which is responsible for the above mentioned results. Employing techniques such as florescent and radioactive labeling could be used to illustrate that *in situ* conversion of $25OHD_3$ into $1,25(OH)_2D_3$ does occur within the tumor. Presently, we are developing a mouse knock out model that will abrogated 1αOHase gene expression within the mouse mammary epithelium using lox P 1-cre recombinase system in the PyVMT model. This will clearly demonstrate that *in situ* conversion of $25OHD_3$ into $1,25(OH)_2D_3$ does occur within the tumor and that the inhibitory affects are mediated through an autocrine mechanism.

## 4.4 Potential Molecular Mechanisms of 1,25-dihydroxyvitamin $D_3$ Action

Based on our observations, the molecular mechanism of action of $1,25(OH)_2D_3$ in preventing the development of mammary carcinomas is complex and likely involves multiple signaling pathways. *In vitro* and *in vivo* data on the anti-tumor activity of $1,25(OH)_2D_3$ indicate that $1,25(OH)_2D_3$ is a putative regulator of several key events in

the cell cycle (336-339). Our *in vivo* observation in this mammary carcinoma model indicates potent inhibitory effect of $1,25(OH)_2D_3$ on the cell cycle progression likely through the regulation of cyclin and cyclin kinase inhibitors as previously described (272-274,340,341). $1,25(OH)_2D_3$ has been shown to induce human breast tumor regression by a mechanism that involves both activation of apoptosis and inhibition of proliferation (337). We are currently examining indices of apoptosis in out model to better define this mechanism. Furthermore, the inhibitory actions of $1,25(OH)_2D_3$ may be also linked to modulation of protooncogenes and tumor suppressor genes such as c-MyC, p21, p27 and Rb (343,344).Studies aimed at examining the expression of these genes in our model are currently underway.

## 4.5 Future Directions and Conclusions

In summary, our study strongly argues for an important autocrine role of 1αOHase expression in breast tumor cells. Accumulation of intra-tumoral $1,25(OH)_2D_3$ in response to $25OHD_3$ accumulation strongly suggests that locally produced $1,25(OH)_2D_3$ plays a significant role in restraining tumor growth in an autocrine manner. More definitive studies on the physiological importance of tumoral 1αOHase will necessitate the use of conditional knock-out models in which 1αOHase is specifically ablated in the mammary epithelium. Such studies are currently underway in our laboratory using lox P 1-cre recombinase system in the PyVMT model.

Nevertheless, our study clearly indicates that the 1αOHase of breast tumor cells can be used advantageously and without significant side effects to inhibit tumor growth and progression. Indeed, our data indicates that $25OHD_3$ is as effective as $1,25(OH)_2D_3$ in inhibiting tumor progression without any evidence of hypercalcemia . We plan to further pursue these studies using combinational therapies with other anti-cancer agents used for

the treatment of breast cancer such as taxol and herceptin. Additionally, we also plan to study in more detail the mechanism of anti tumor activity of $1,25(OH)_2D_3$ in this model including cell cycle regulation, angiogenesis and metastatic markers. We also plan to examine the potential benefit of $25OHD_3$ administration in models of skeletal metastasis. In summary our study points to promising new strategies in the treatment of breast cancer and its complications.

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