1VITAMIN D METABOLITES INHIBIT ADIPOCYTE DIFFERENTIATION IN 3T3-L1 PREADIPOCYTES.

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DEDICATION

To my loving husband Prabhu and my dear daughter Sneha for their constant love and support.

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CHAPTER I

LITERATURE REVIEW

A. Introduction

The rising prevalence of obesity in the past 20 years underscores the need to understand the biology and key regulatory processes involved in the development of obesity. Adipogenesis, or the formation of new adipocytes from precursor cells, is one such regulatory process. Fully mature, differentiated adipocytes accumulate lipid and contribute to fat cell mass (18). Although vitamin D generally promotes cell differentiation, its role in adipocyte differentiation and proliferation is less clear. Vitamin D circulates as 25-hydroxyvitamin D (25-D), which is converted to the vitamin's active form, 1,25-dihydroxyvitamin D (1,25-D) by the renal 1α-hydroxylase (17, 31). Recent studies indicate that this activating enzyme 1α-hydroxylase is also expressed in non-renal tissues such as placenta, prostate, colon, breast (16) but expression of the 1-α hydroxylase has not been described in adipocytes. Epidemiologic studies show a negative correlation between serum levels of 25-D and adiposity, and cell culture studies suggest that 1,25-D may influence adipogenesis (20, 32). The murine 3T3-L1 preadipocyte cell line has been widely used to study the adipogenic process (23,19). Recent findings in this cell line indicate that 1,25-D inhibits differentiation of preadipocytes to mature adipocytes (13,3,7,24). Furthermore, 1,25-D exerts its effect through binding with the nuclear receptor VDR (13,3). However, no

studies have investigated the role of 25-D in these cells. This research is designed to investigate the roles of both 25-D and 1,25-D in adipocyte differentiation in murine 3T3-L1 cells and to determine whether these cells express the 1α-hydroxylase.

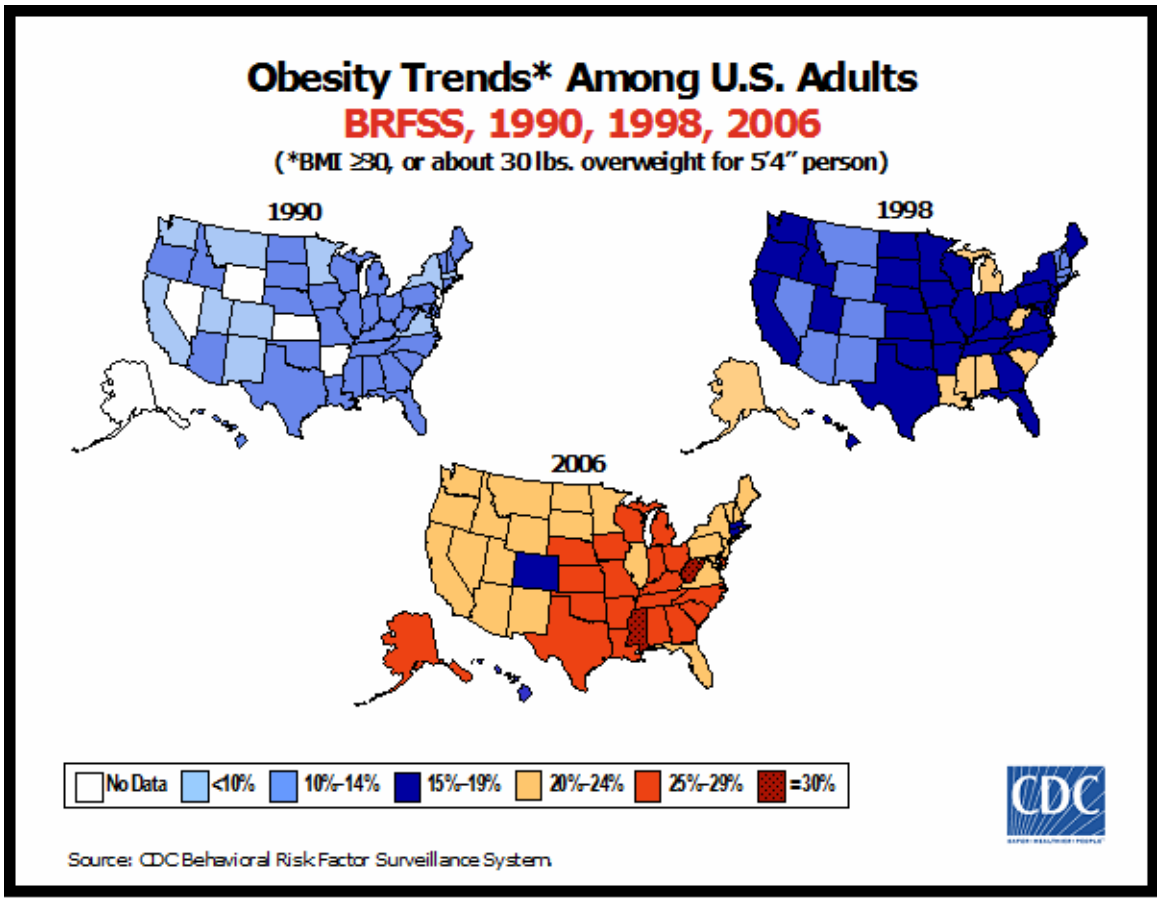
B. Overview of Obesity

The growing prevalence of obesity is one of the biggest public health concerns in the US and around the world. There has been a sharp rise in obesity over the past 20 years (40, 41, 42). Figure 1, shows the growing trends in obesity in the US population. In the 1990s the Behavioral Risk Factor Surveillance System (BRFSS) data shows 10 states with <10% obesity and no state having >15% prevalence in obesity. In comparison there has been dramatic rise in obesity prevalence 2006 to only 4 states with <20% obesity and 22 states with a prevalence of $\geq$25% obesity of which 2 states had >30% prevalence of obesity (39).

Obesity is defined as body mass index (BMI) $\geq$ 30. The number of people in the obese and morbidly obese (BMI>40) category has seen a dramatic increase since 1990 (41,42, 40). Obesity increases an individual's risk to comorbidities like type 2 diabetes, cardiovascular diseases and certain cancers (41,51). Obesity also increases an individual's rate of mortality from acute or chronic disease. Obesity is a result of a constant increase in energy intake and lower output. There are many factors contributing to obesity including high energy intake, decreased physical activity, sedentary lifestyle, increase in fast

food etc (42). However, understanding the science behind obesity is an important aspect in the prevention of this disorder. Over the past decade there have been a number of studies focusing on the science of obesity and the key regulatory processes involved in the development of obesity.

Figure: 1. Obesity trends in the US. This figure shows the growing trends in obesity in the US population. In the 1990s the BRFSS data show 10 states with <10% obesity and no state having >15% prevalence in obesity. In comparison there has been dramatic rise in obesity prevalence 2006 to only 4 states with <20%obesity and 22 states with a prevalence of ≥25% obesity of which 2 states had >30% prevalence of obesity.



C. Adipose Tissue

Obesity very simply can be defined as an unhealthy increase in the adipose tissue mass. In humans, adipose tissue is of two types brown and white. The role of both are opposite of each other.

Brown adipose tissue (BAT): It is present in significant amounts in infants and is negligible in adults. It gets its color due to the high number of mitichondria and the rich blood flow through it. It contributes to thermogenesis which is the involuntary heat production or release of energy through physical activities like shivering etc in response to cold (45). BAT consists of a protein in its inner mitochondrial membrane called thermogenin also called uncoupling protein-1 (UCP-1) which dissipates energy through the uncoupling of energy yielding nutrients and ATP production (45). UCP-2 and UCP-3 are the other uncoupling proteins identified so far in other tissues of the body as well (47).

White adipose tissue (WAT): White adipose tissue primarily stores energy as triacylglycerols. Majority of adipose tissue in our body is white adipose. They are present as subcutaneous fat and visceral fat. The body can store limitless amount of fat. Adipose tissue is made up primarily of adipocytes. Adipocytes are rich sources of energy made up of 80% lipid and 20% of water and protein (50). They are capable of growing 50 times in weight (50). In addition to adipocytes the adipose tissue also consists of macrophages, preadipocytes, fibroblasts, loose connective tissue and various cell types (43).

1. Functions of Adipose Tissue

Energy Storage: One of the main functions of the adipose tissue is in energy storage. Excessive energy is stored as triacylglycerols in the adipose tissue. During starvation, this can be mobilized for energy.

Insulation: Fat layer beneath our skin serves as an insulating layer.

Protection: Many important organs are surrounded with a layer of fat which protects it from injury.

Adipose tissue produces and regulates certain cytokines which are proteins called adipokines. These proteins are mediators, which act as hormones and they are called adipocytokines / adipokines. These adipokines have been implicated for their role in different mechanisms like regulation of energy balance, insulin action, glucose metabolism, inflammation and immunity (43). Some of the adipokines being studied are leptin, adiponectin, resistin, visfatin, TNF-α etc. Leptin is known to regulate appetite, food intake and energy expenditure. Adiponectin plays a beneficial role in insulin resistance, atherosclerosis, inflammation and dyslipidemia (52). Similar to adiponectin, visfatin also plays a beneficiary role in glucose metabolism by affecting the insulin signaling pathways (53). TNF-α is known for its pro inflammatory role. In general adipokines play a dual role of friend and foe in obesity and development of obesity related comorbidities (51).

2. Development of Obesity and Adipogenesis

Obesity is a state of positive energy balance leading to increase in adipose tissue mass. Adipose tissue mass increase relates to the changes in the size or number of adipocytes.

Adipose tissue mass can increase in two ways: hyperplasia, which is characterized by an increase in the cell number or hypertrophy, which is characterized by an increase in the size of the cell (19).

Adipocytes develop from precursor cells, called preadipocytes, which are fibroblast-like cells. Adipogenesis is defined as the formation of round lipid-filled adipocytes from preadipocytes and is one of the key regulatory processes involved in the development of obesity (3). These newly formed cells can further increase in size by accumulating more lipid droplets. When these adipocytes exhaust their storage capacity, there is a consequent increase in cell number (19).

Thus, both increase in cell number and cell size contribute to adipose mass. Adipogenesis occurs throughout the lifetime in response to normal cell turnover and to excess energy to be stored as fat mass (48).

3. Adipocyte Development

The adipose lineage originates from multipotent mesodermal stem cells (19). The precursor cells are present in the stroma of the adipose tissue which

can undergo commitment to the adipocyte lineage through a multi-step process to form preadipocytes (19). These preadipocytes can undergo terminal differentiation under an adipogenic hormonal milieu to form mature fat-filled adipocytes. These mature adipocytes can express adipogenic markers such as fatty acid synthase (FAS), lipoprotein lipase (LPL), acetyl-CoA carboxylase, glucose transporter 4 (GLUT4), fatty acid binding protein (aP2) and have the capacity to accumulate large quantities of triacylglycerol (13).

The adipocyte differentiation occurs in a series of well defined steps.

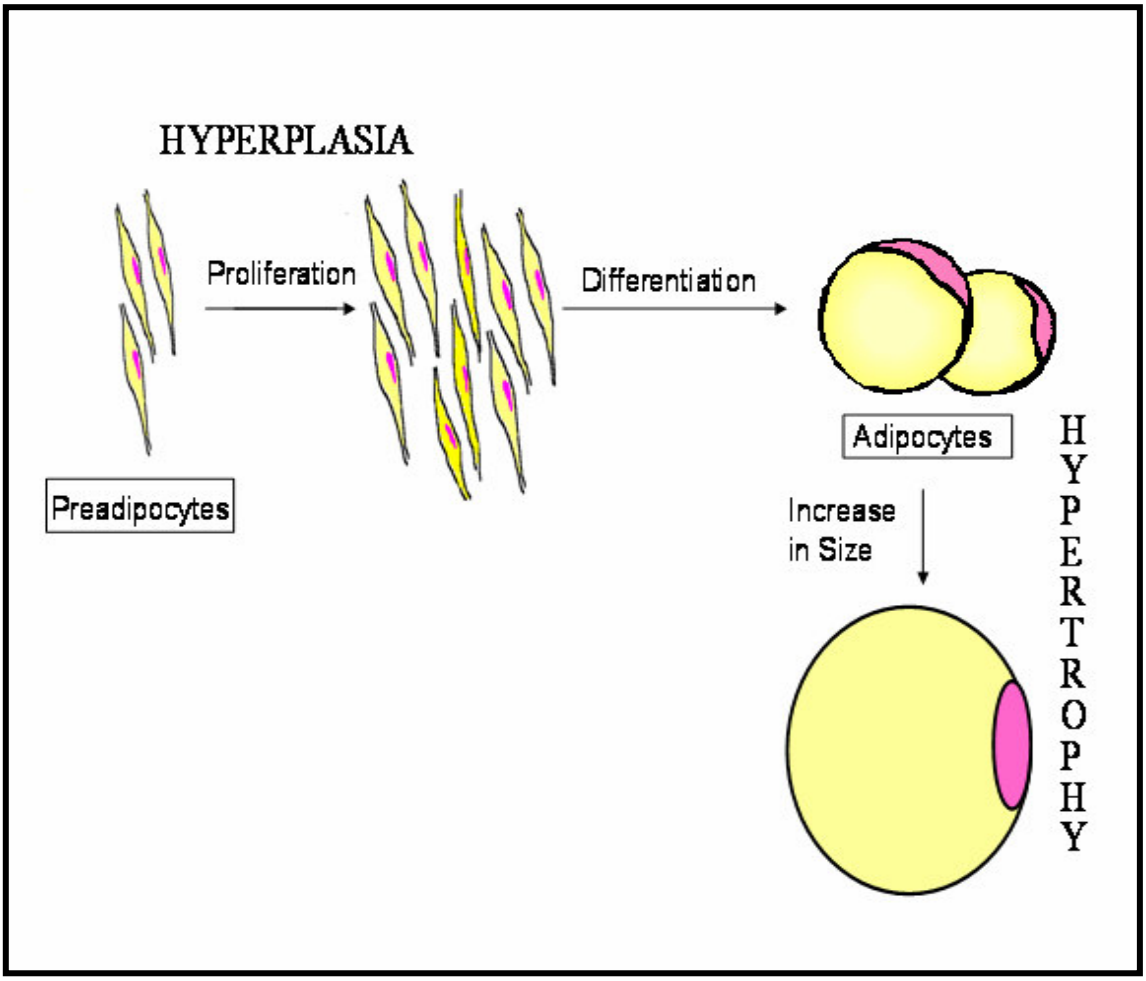
Commitment: In this phase, pluripotent mesenchymal stem cells undergo determination to the adipose lineage in response to certain signaling events (19), although It is unclear what actually are these signaling factors. It is speculated that it may be a gene or the mature adipocyte which secretes some signaling molecule (19,23). The preadipocytes are formed in this phase. It is suggested that BMP4 could possibly be the signal for this determination (23).

Recruitment and Proliferation of adipocytes: In this phase, the preadipocytes proliferate and increase in numbers. The preadipocytes undergo a growth arrest phase after which they undergo 2 more rounds of proliferation before the next phase (19). This is called mitotic clonal expansion. It is controversial if mitotic clonal expansion is absolutely necessary for terminal differentiation. However, certain important transcriptional factors are generated during this phase which regulate the terminal differentiation to mature adipocytes (19,23).

Terminal Differentiation: This is the final phase where the preadipocytes undergo the final step to form the mature lipid filled adipocyte. These adipocytes

produce proteins required for lipid transport and synthesis, insulin sensitivity and other adipocyte specific proteins (23). On introduction of hormonal inducers of differentiation such as insulin, dexamethasone and 3-isobutyl-1-methylxanthine, the preadipocytes express a cascade of transcriptional factors, coactivators and cell-cycle proteins which trigger the necessary steps to induce terminal differentiation (19). The differentiation process is complex and under the regulation of multiple inhibitory and stimulatory signals. Figure 2 shows the steps involved in adipocyte differentiation. After determination to the adipocyte lineage, the preadipocytes increase in number and undergo differentiation to mature adipocytes, which then can accumulate triacylglycerol and increase in size.

Figure: 2. Adipocyte Development. This figure shows the steps involved in adipocyte differentiation. After determination to the adipocyte lineage the preadipocytes increase in number and undergo differentiation to mature adipocyte, which can accumulate triacylglycerol and increase in size.



4. Adipogenic Transcription Factors

The most important transcriptional factors involved in the regulation of differentiation are the PPAR family and the CCAAT/enhancer-binding proteins. Peroxisome proliferators-activated receptors (PPARs) family:

They belong to the nuclear receptor family like retinoic acid receptors and vitamin D_3 receptors. There are 3 members of the PPAR family α, β and γ (19). The PPARs have a ligand independent transactivation domain, DNA binding domain and a ligand binding, a dimerization domain with a ligand-dependent transactivation domain (19). PPAR forms a heterodimer with RXR prior to binding of DNA to the peroxisome proliferators-activated receptor element in the promoter region of the target genes. Of the different members of PPAR family the chief regulator of adipogenesis is PPARγ (19).

PPARγ : It is the key regulator of adipogenesis. It is important for promotion of differentiation to the mature adipocyte and for maintenance of the differentiated state (19, 23). It is expressed during differentiation and is responsible for activation of adipogenic genes. Some of the genes activated are aP2, lipoprotein lipase, acyl CoA synthase and phosphoenolcarboxykinase. These genes are involved in fatty acid transport, uptake and storage. Studies have demonstrated the importance of PPARγ in the adipogenic process. Overexpression of PPARγ in vitro induces adipocyte differentiation in fibroblasts (19, 23). PPARγ is required for adipogenesis and no factor has been identified which promotes adipogenesis in the absence of PPARγ (23). Thiazolidinedione (TZD), an anti-diabetic drug is a

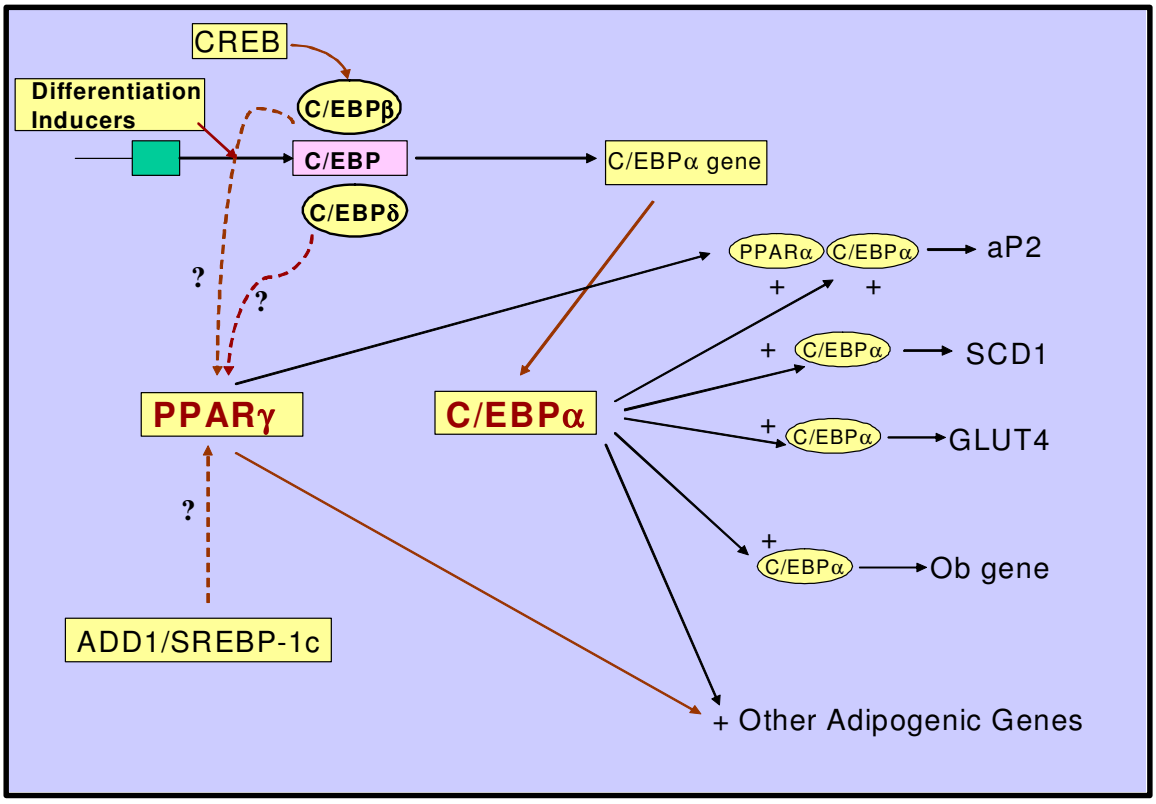
ligand for PPARγ (23). Studies conducted so far have not identified an endogenous ligand for PPARγ (23). PPARγ has 3 isoforms PPARγ1, γ2 and γ3. PPARγ1 and γ2 are expressed in adipocytes are important regulators of adipogenesis (19). Few studies have identified PPARγ2 as the main isoform required for adipogenesis, while others have suggested both are required for adipogenesis (19). The CCAAT/enhancer-binding protein C/EBPβ and C/EBPδ are required for the activation of PPARγ expression (23,19). However their role is unclear. Sterol response element-binding protein-1c (SREBP1c) can also influence the activation of PPARγ (10). It is understood that PPARγ along with C/EBPα are responsible for activation of many adipogenic genes involved in adipogenesis (19).

CCAAT/enhancer-binding Proteins (C/EBPs):

They are involved in promotion of adipogenesis. They consist of 6 isoforms α, β, γ, δ, ζ(CHOP-10), and ε. All except the ε form is involved in adipogenesis (19). C/EBPβ and δ are involved in the early regulation of the adipogenesis. cAMP response element binding protein (CREB) is responsible for the transcriptional activation of C/EBPβ (19). Addition of differentiation inducers like insulin and cAMP leads to the phosphorylation of CREB and its effects early in the differentiation process. C/EBPβ is required for the mitotic clonal expansion to occur (19). The C/EBPβ and δ is responsible for activation of C/EBPα (19). Once expressed, C/EBPα continues to be active by an autoactive mechanism (19).

C/EBPα is essential for differentiation and it activates several adipogenic genes (19). Cell culture study has shown that blocking of C/EBPα inhibit adipocyte differentiation (19). Studies conducted on C/EBPα (-/-) mice shows defects in accumulation of lipid (19). It is expressed after the clonal expansion phase in differentiation as it has anti-mitotic activity (19). Other C/EBP isoforms CHOP and C/EBPγ are anti-adipogenic (23).

Figure 3: Transcription factors and adipogenic genes during Differentiation. This shows the expression of the various transcription factors after induction of differentiation leading to expression of the adipogenic genes. The dotted lines signify a potential activation of the particular factor. CREB – cAMP response element binding protein, SREBP-1c – sterol regulatory element binding protein 1c, SCD1 – stearoyl- CoA desaturase 1, aP2- adipocyte protein 2 a fatty acid binding protein, GLUT4 – glucose transporter and OB gene – the obese gene.



(**Modified from**: Otto TC, Lane MD. Adipose development: from stem cell to adipocyte. Crit Rev Biochem Mol Biol 2005;40:229-42.)

5. Cell Culture Models

Much of our knowledge about adipogenesis or fat cell biology has been through in-vitro experimental cell culture model systems. There are 2 types of cell culture models:

Primary Culture: They are isolated directly from vascular stroma of adipose tissues from humans or animals e.g. human preadipocytes (phPA), pigs and rodents (19,18). These cells are difficult to isolate and have a limited life-span. They are also expensive. Furthermore, these cells are heterogenous which means they do not exhibit similar characteristics after each passage (9). This cell model can be used to confirm the results seen in cell line models.

Cell Lines: They are of two types: multipotent fibroblastic cell lines which are not committed and have adipogenic potential, e.g. NIH-3T3, Balb/c3T3, C3H 10T1/2 etc (9) and established preadipocyte cell lines which have undergone commitment to the adipose lineage (9). Examples of these cell lines are 3T3-L1, Ob1771, 3T3F442A, TA1, Ob17 etc. They exhibit morphology and biochemical properties similar to in vivo adipocytes. They are homogenous and have a long life span in culture (9).

3T3-L1 preadipocytes: The murine 3T3-L1 preadipocyte cell line is a widely used model to investigate the adipocyte differentiation process. They are derived from cloned subline of Swiss 3T3 mouse embryo fibroblasts (40). This model was developed by Howard Green and colleagues (40). They can undergo differentiation in culture and exhibit morphology and biochemical properties similar to adipocytes. Furthermore, when transplanted subcutaneously into BALB

C (athymic) nude mice these preadipocytes differentiate and develop into tissue similar to adipose tissue (25). The 3T3-L1 preadipocytes can grow exponentially in culture and when fully confluent reach a growth arrest and enter a stable resting state (40). The post confluent 3T3-L1 preadipocytes when treated to a hormonal cocktail of differentiation inducers consisting of dexamethasone, insulin and 3-isobutyl-1-methylxanthine (MIX) which increases cAMP can differentiate to form mature lipid-filled adipocytes (28). This hormonal cocktail is abbreviated as MDI (25). The preadipocytes differentiate to mature adipocytes between 4 to 8 days.

6. Hormonal Inducers of Adipogenesis

Adipocyte differentiation is influenced by endocrine signals (9). The hormonal inducers of differentiation in most cell systems are insulin, IGF-1, growth hormone, glucocorticoid and cAMP. In 3T3-L1 cells a mixture of insulin, dexamethasone (which works as a glucocorticoid), fetal bovine serum (which supplies the growth hormone) and 3-isobutyl-1-methylxanthine called MIX (increases intracellular cAMP) induce differentiation in postconfluent preadipocytes.

Insulin/IGF-1: Insulin promotes adipocyte differentiation. Preadipocytes hardly express insulin receptors but have IGF-1 receptors. Insulin mediates its adipogenic actions in preadipocytes through the IGF-1 receptor signaling. However, as the differentiation progresses and mature adipocyte is formed they

express more insulin receptors than IGF-1 receptors (48, 9, 23). Insulin and IGF-1 activate several signal transduction pathways which leads to the adipogenic effects exerted by them (48,23).

Glucocorticoids: Glucocorticoids are stimulators of differentiation especially in 3T3-L1 cells. Dexamethasone (Dex) functions as the glucocorticoid and exerts its adipogenic effects through the glucocorticoid receptor (GR). Dex appears to induce the expression of C/EBPδ which causes its adipogenic activity (48). However, this effect is unclear as studies have demonstrated that overexpression of C/EBPδ in cells still require Dex for adipogenesis signifying another mechanism through which Dex operates (48). Another potential role of glucocorticoids is inhibition of phospholipase A2. This inhibition leads to decreased prostaglandin induced inhibition of differentiation (9).

Growth Hormone: Growth hormone has been shown to promote adipogenesis in preadipocyte cell lines, but appears to not have the same effect in primary cell lines (9). In 3T3-L1 cells fetal bovine serum provides the growth hormone required for differentiation.

cAMP: The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine acts as the agent that increases intracellular cAMP levels (48, 9).

D. Vitamin D

Vitamin D is a seco-steroid hormone and is well-known for its classic role in calcium and phosphorus homeostasis. It is a fat soluble vitamin and deficiency of which causes bone deformities in children called rickets and in adults causes osteomalacia. Unlike typical vitamins which need to given in small amounts through the diet or supplements, vitamin D can be synthesized endogenously through photolysis of 7-dehydrocholesterol in the skin.

1. Cutaneous Synthesis of Vitamin D

Vitamin D is synthesized in the body either from sunlight or from dietary sources. The UVB rays with wavelengths 290 and 315nm from the sunlight acts on 7-dehydrocholesterol present on the skin to make previtamin D3. Previtamin D gets converted to Vitamin D by thermal transformation. Prolonged exposure to sunlight can lead to conversion of previtamin D to inactive catabolic compounds called lumisterol and tachysterol (2). Prolonged sun exposure can also cause vitamin D to degrade to suprasterol I, suprasterol II and 5,6-trans-vitamin D (2). The newly formed Vitamin D enters the circulation and binds to a specific vitamin D binding protein called α_1-globulin.

2. Activation of Vitamin D

Vitamin D bound to α_1-globulin travels to the liver where it gets hydroxylated at carbon 25 by 25-hydroxylase (CYP27A) to form 25-hydroxyvitamin D (25-D). This form is the main circulating form of the vitamin. The normal range for 25-D in the blood is between 20-87 nmol/L and it has a half life of 3 weeks (2). The 25-hydroxyvitamin D further undergoes hydroxylation either at the 1 position by renal enzyme 1α-hydroxylase (CYP27B1). Renal 1α-hydroxylase causes hydroxylation to 1,25-dihydroxyvitamin D. This is the active form of vitamin D and is capable of producing the functional effects of vitamin D. The ubiquitous enzyme 24-hydroxylase (CYP24) can inactive both vitamin D metabolites, it converts 25-D to 24,25-dihydroxyvitamin D and 1,25-D to 1,24,25 $(OH)_3$ (calcitetrol). These metabolites are further degraded and excreted out as calcitroic acid. CYP24 expression is regulated by 1,25-D levels (27). Vitamin D from the diet enters the circulation directly and can undergo similar hydroxylations in the liver and kidneys. Vitamin D from dietary sources are taken up by enterocytes and incorporated into chylomicrons. These chylomicrons enter the venous blood stream after being released by the enterocytes into the lymphatic system and then drained into the blood stream (2). The chylomicron remnants reaches the liver where the vitamin D is hydroxylated to 25-D by 25-hydroxylase and enters the circulation bound to vitamin D binding protein (17). It can undergo further hydroxylation in the kidney. Figure 4, shows the various vitamin D metabolites derived through cutaneous synthesis and diet to the formation of the 25-hydroxyvitamin D_3 (circulating form) and its conversion to

1,25-dihydroxyvitamin D_3 (active form) and their respective inactivation to calcitroic acid. Also shown are the major enzymes involved in the activation and inactivation of these metabolites.

3. Dietary sources of vitamin D

Fortified milk, eggs from hens fed vitamin D-fortified feed, fatty fish, fish oils, and animal liver (2).

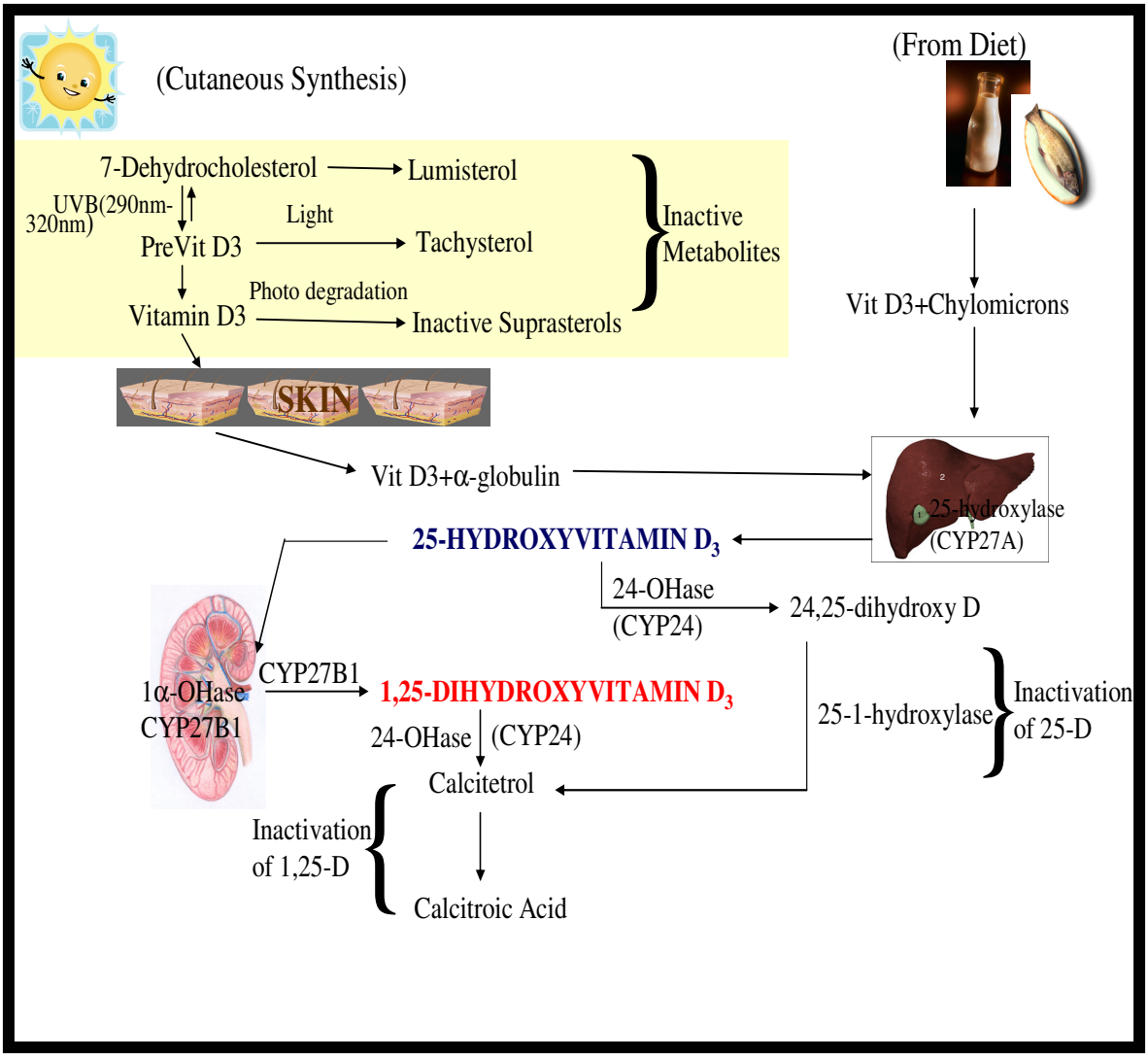
RDA/AI: Vitamin D does not have a set RDA because an estimated average requirement (EAR) could not be set due to results being confounded by sun exposure. An adequate intake (AI) is set for vitamin D. The AIs are as follows:

Infants to 50 years old → 200IU/day (5µg)

51 to 70 years old → 400 IU/day (10µg)

Over 70 years → 600 IU/day (15µg)

Figure: 4. Vitamin D Metabolites. This shows the various vitamin D metabolites derived through cutaneous synthesis and diet to the formation of the 25-hydroxyvitamin D_3 (circulating form) and its conversion to 1,25-dihydroxyvitamin D_3 (active form) and their respective inactivation to calcitroic acid. The major enzymes involved in the activation and inactivation of these metabolites. CYP27A – 25-hydroxylase, CYP27B1 - 1α-hydroxylase and CYP24 – 24-hydroxylase.



4. Factors Affecting Vitamin D Synthesis

Vitamin D synthesis from sunlight is affected by various factors. The following are some of the factors affecting its synthesis from sunlight:

(i) Clothing: It blocks cutaneous vitamin D synthesis.

(ii) Latitude: During the months from November through February people in northern latitudes do not make vitamin D from sun exposure. This is because the angle of the sun rays blocks the wavelengths required for conversion of 7-dehydrocholesterol to previtamin D. Hence no cutaneous synthesis of vitamin D occurs (46).

(iii) Skin tone: Melanin the skin pigment acts as a natural sunscreen, hence people with darker skin require much more sun exposure than light skinned people for cutaneous vitamin D synthesis.

(iv) Sunscreen: Sunscreens contain SPF-8 which blocks UV B rays required for its synthesis by the skin.

(v) Age: Aging causes a decrease in vitamin D synthesis, absorption and metabolism. Aging can cause a decrease in synthesis of vitamin D by skin due to decreased 7-dehydrocholesterol levels. Hence there is a decrease in serum levels of 25-D. Aging can also lower levels of intestinal vitamin D receptors (46).

(vi) Temperature: Prolonged sun exposure and increased skin temperature leads to formation of vitamin D degradation products.

(vii) Heredity: Vitamin D polymorphisms also decrease vitamin D formation.

5. Vitamin D Metabolism

The main function of vitamin D is maintaining the calcium and phosphorus levels in the blood. Vitamin D level is regulated by PTH, 1,25-D (calcitriol) and serum calcium and phosphorus levels.

Parathyroid Hormone - PTH is secreted by the parathyroid glands located in the hypothalamus. The parathyroid glands have cell surface calcium sensors which can detect low levels of calcium in the blood. PTH is released in response to hypocalcemia. Increase in PTH levels is sensed by PTH sensors in the kidney (2). Once PTH is released it acts on the kidneys by increasing the activity of 1α-hydroxylase which causes increased production of 1,25-D (calcitriol) from 25-D. 1,25-D brings about the effects of increasing serum calcium levels through the intestine and bones.

Calcitriol (1,25-D) – It is the active metabolite of vitamin D and it's main functions is to regulate serum calcium levels by affecting the intestine and bone.

Intestine - Calcitriol directly increases intestinal absorption of calcium by increasing the activity of proteins like calbindin. Calbindin is responsible for the transport of calcium across the intestinal mucosa. It also increases activity of other proteins like alkaline phosphatase, calmodulin, low-affinity calcium-dependent ATPases, brush border actin (17).

Bone- Calcitriol can indirectly affect osteoclastic activity and increase serum calcium levels through bone resorption. Calcitriol responsive vitamin D receptors are present on osteoblasts. 1,25-D mobilizes bone to release calcium through its action on osteoblasts. Calcitriol enhances the production of osteoclast

sensitive cytokines like IL-6 and IL-12 which can regulate osteoclastic activity and bring about bone resorption (17). In turn, calcitriol levels can negatively regulate PTH. Low levels of calcitriol increase PTH secretion whereas high levels inhibit PTH secretion (2). Further, high 1,25-D levels downregulates the activity of renal 1α-hydroxylase and upregulates 24-hydroxylase activity (27). This brings about the catabolic breakdown of 1,25-D to 1,24,25-D (calcitetrol) can occur.

1α-hydroxylase – It is the renal enzyme involved in the activation of 25-D to 1,25D. 1,25-D negatively regulates the renal 1 α-hydroxylase activity. Disruption of the 1 α-hydroxylase gene in null mutant mice results in hypocalcemia, hyperparathyroidism, growth retardation, osteomalacia and reproductive dysfunction (27). Recent studies have demonstrated the presence of 1α-hydroxylase in extra-renal tissues like immune cells, breast, pancreas and the prostate (16). In the extra renal tissues this enzyme is influenced by cytokines (37,15). 1,25-D regulates cell differentiation function in certain tissues and the presence of 1α-hydroxylase in these tissues could play an important role in the paracrine regulation of 1,25-D (15).

6. Mechanism of Action of Vitamin D

Vitamin D exerts its metabolic responses either by rapid response or non-genomic responses of 1,25-D or through genomic response.

Genomic Responses: It is also called less rapid or genome-initiated responses. These responses can take minutes to hours or days. The genomic responses of 1,25-D is mediated through the nuclear vitamin D receptor (VDR) (8). 1,25-D binds to VDR on the nucleus of the cell and binding to the ligand brings about a conformational change and facilitates the formation of a heterodimer with retinoid X receptor (RXR) (8). This further causes phosphorylation to activate binding to the VDRE (vitamin D receptor element) in the promoter region of target genes and causes the upregulation of vitamin D responsive genes thereby causing the tissue specific responses (8). Some of the genes upregulated by 1,25-D are calbindin D, osteocalcin, osteopontin, plasma membrane calcium pump, interleukin-1, interleukin-6 (2, 8).

VDR: It is a 50 kDa nuclear receptor which has a high affinity to 1,25-D. It is a member of the nuclear receptor family of ligand activated transcription factors (27). It was first found in the intestinal mucosa, but now is found in other tissues like the kidneys, bone, parathyroid glands, β-cells of the pancreas, placenta, pituitary, uterus, mammary glands, skin, thymus, monocytes, macrophages and T lymphocytes (2). The primary amino acid sequence of VDR has 5 functional domains which include regions for nuclear localization, DNA binding, heterodimerization, ligand binding and transcriptional activation (17). Regulation of gene transcription is dependent on the ligand binding to the

receptor (17). An unoccupied VDR does not bring about the gene transcription as that of a VDR bound to its ligand 1,25-D. Shape of the ligand is also an important aspect for the up or downregulation of the genomic responses. VDR bound to the hat icon shape of 1,25-D leads to the appropriate gene responses (17).

Non-Genomic or Rapid responses: It is also called rapid responses as it takes a few seconds or minutes for the tissue specific responses. These responses are mediated by a cell membrane receptor for vitamin D called MARRS (membrane activated rapid response steroid binding protein) (44). The binding of the 1,25-D to the cell membrane receptor causes a number of signal transduction pathways which leads to the corresponding physiological response (8). In enterocytes, ligand binding to the receptor causes G-protein activation leading to phospholipase C linked hydrolysis of phosphatidylinositol bisphosphate (PIP2). This causes release of diacylglycerol and inositol triphosphate (IP_3). IP3 leads to the opening of calcium transport channels in the ER leading to the release of Ca+ ions which activates calcium dependent proteins like calmodulin. This brings about the appropriate physiological responses. Diacylglycerol leads to activation of protein kinase C and mitogen activated (MAP) kinases which in turn also causes the physiological responses. These physiological responses include transcaltachia, activation of voltage-gated calcium channels, increase in intracellular calcium, induction of phospholipids and sphingolipid turnover (2).

7. Non-Classical Functions of Vitamin D

Cell differentiation and antiproliferation: 1,25-D promotes terminal differentiation of keratinocytes and inhibits proliferation and this has been used in the treatment of psoriasis (2). In vitro studies, vitamin D has been shown to suppress cancer through cell cycle arrest and growth inhibition of the proliferating cancer cells (4). 1,25-D downregulates inflammatory markers such as IL-2 and IL-12 and exerts its antiproliferative effect (15). Cancer research has shown the presence of VDR, 1 α-hydroxylase (CYP27B1) and 24-hydroxylase (CYP24) in colon, prostate and breast cancer cells. It has been suggested that the local production of 1,25-D in these cells are responsible for the antiproliferative effects (34). Furthermore, in MCF-7 human breast cancer line the malignant cells regulate 24-hydroxylase expression and through this mechanism protect itself from the anti-cancer effects of 1,25-D (34).

Endocrine glands: Vitamin D has been implicated in the pathogenesis of type 1 diabetes (35). Vitamin D may play a protective role in type 1 diabetes possibly due to its role in β-cell function and normal insulin secretion (35,15). It is required for normal insulin secretion. 1,25-D can alter T-cell differentiation and induce cytokine secretion. Studies in NOD mice, an animal model for type 1 diabetes shows that vitamin D deficiency early in life causes type 1 diabetes (35).

Immune system: 1,25-D has potent immunomodulatory effects and may have a preventive effect in autoimmune deficiency (15). A recent study has a demonstrated a strong inverse correlation between vitamin D levels and multiple sclerosis (MS), a autoimmune characterized by demyelination of central nervous

system (36). This is further supported by 1,25-D inhibiting autoimmune encephalomyelitis, an animal model for MS (36,15).

E. Vitamin D and adiposity

To understand the role of vitamin D in obesity it is important understand correlations at the population levels. Epidemiologic studies conducted on different populations have examined the role of vitamin D and adiposity. Both metabolites of interest 1,25-D and 25-D have been studied in relation to obesity. 25-D which is the circulating form of vitamin D is negatively correlated with body fat content and BMI (20,32). 1,25-D has shown inconsistent results in some studies suggesting higher levels in obese individuals (20). However, a more recent study has noted lower 1,25-D levels in obese as compared to normal weight individuals (20). It was also noted that these individuals had higher iPTH levels which can be attributed due to low 1,25-D levels (20). The study suggested that a low serum 25-D level was due to decreased availability of vitamin D and their increased sequestration in fat. Although the iPTH levels were high there still was lower levels of 1,25-D. The study suggests that the decreased 25-D levels as a possible reason (20). However, 1,25-D has a very short half life in the blood and is not a reasonable marker of an individual's vitamin D status. Obese African-Americans showed higher prevalence of hypovitaminosis D indicating race as another factor in this correlation (32). A study of preoperative morbidly

obese patients scheduled for bariatric surgery found 62% of them to have 25-D levels below normal and those with normal levels were in the lower quartile (49). There is substantial evidence which supports that obese individuals have a compromised vitamin D status in comparison to lean individuals.

F. Vitamin D and adipocyte differentiation

Preosteoblasts and preadipocyte arise from common progenitor mesenchymal stem cells in the bone marrow (29, 12). Vitamin D influences bone osteoblast differentiation (29,12). It is hence possible that 1,25-D could share common mechanisms and play an important role in adipocyte differentiation too (29).

The inhibitory role of 1,25-D on adipocyte differentiation was first demonstrated 20 years ago in 2 different studies (24,10). These studies were conducted using 3T3-L1 preadipocytes and 1,25-D treated cells showed a 50% decrease in the triglyceride accumulation as compared to the control (24,10). Similar results were also seen in ST-13 preadipocyte cell line (24). These studies predicted a receptor-mediated action of 1,25-D on preadipocyte differentiation inhibition (24). However, studies done on rat calvaria cells and rat bone marrow cells suggested an opposite role for 1,25-D in the differentiation process suggesting that it promoted differentiation in these cells (1). Similar studies conducted on primary human adipocytes demonstrated that 1,25-D

stimulates fatty acid synthase (FAS) and suppresses UCP-2 and leptin production (26, 13). Another study by Vu et al (12) showed an increase in LPL expression in the cells and media followed by an increase in the expression of the adipogenic gene aP2 in 1,25-D treated 3T3-L1 cells. Despite conflicting results in other cell models, research involving 3T3-L1 preadipocytes demonstrated mostly consistent results for1,25-D as an inhibitor of adipocyte differentiation (7, 3, 13, 10, 24).

Research by Hida et al (7) highlighted some of the molecular mechanism that were involved in 1,25-D linked inhibition.The study showed that 1,25-D inhibited adipocyte differentiation by inhibiting PPARγ2 expression suggesting that 1,25-D inhibits adipocyte differentiation in the early stages (7). However, 1,25-D concentration used in this study was 1μM which is very high (7). The identification of the vitamin D response element in the promoter region of Insig-2 (insulin induced gene-2) highlights another novel mechanism for the inhibitory effects of 1,25-D. Insig-1 and Insig-2 are proteins present in the endoplasmic reticulum which can block sterol regulatory element-binding proteins (SREBPs) (14). SREBPs are required for transcriptional activation of synthesis of cholesterol and fatty acid. They can also block adipogenesis and lipogenesis (14). The study by Lee et al identified the presence of VDRE in the promoter region of Insig-2 which binds to the VDR and RXR heterodimer and activates the 1,25-D mediated effects (14). They hypothesized that in addition to blocking transcription of PPARγ, 1,25-D further blocks adipogenesis by upregulation of Insig-2 (14). 1,25-D mediated upregulation of Insig-2 could also potentially have effects on

lipogenesis in mature adipocytes as SREBPs are required for lipogenesis (14). Two recent studies have conducted a detailed analysis on the molecular mechanisms involved in the 1,25-D induced inhibition in 3T3-L1 cells (13,3). The following are some of the important observations demonstrated by these studies. 1,25-D inhibits 3T3-L1 adipocyte differentiation in a dose dependent manner and is time sensitive (3,13). This suggests that 1,25-D can affect differentiation if introduced early in the differentiation program. VDR is expressed early on in adipogenesis and the VDR levels decrease with the progress of differentiation (13, 3). Mature adipocytes do not express VDR. It is the liganded VDR that can inhibit differentiation. This is the potential reason for the time-sensitive inhibition by 1,25-D. Furthermore, expression of major adipogenic genes associated with early and late stages of differentiation like PPARγ, C/EBPα, LPL and aP2 were blocked by 1,25-D in a dose dependent manner. Genes like SREBP-1c and fatty acid synthase (FAS) which are upregulated in mature adipocytes were also suppressed by 1,25-D (13). VDR expression is critical for 1,25-D inhibition as VDR binding to 1,25-D mediates most functions of 1,25-D. Kong et al studied this aspect by differentiating VDR+/+ and VDR -/- mouse embryonic fibroblasts. 1,25-D treatment blocked adipogenesis in VDR+/+ cells but failed to do so in VDR-/- cells (13). Kong et al (13) have demonstrated that direct suppression of PPARγ and C/EBPα by 1,25-D is the main reason for the inhibition. They have further explained that 1,25-D and PPARγ both require RXR binding for their action and as VDR binds to RXR early in adipogenesis there is very less RXR for the PPARγ activation (13). However, Blumberg et al (3) have demonstrated that 1,25-D

suppresses C/EBPβ expression which indirectly downregulates PPARγ and C/EBPα expression and hence the inhibition of adipogenesis. They have also demonstrated that 1,25-D upregulates ETO/MTG8 which is a corepressor for C/EBPβ which could further cause the inhibition (3). However, the role of C/EBPβ in upregulation of PPARγ and C/EBPα is not clearly understood and is under much debate. Both studies have demonstrated a dose dependent and time sensitive 1,25-D induced inhibition of adipocyte differentiation at concentrations ranging from 1nmol/l to 100nmol/l (3,13). The studies also demonstrate different roles for the unliganded VDR. Blumberg et al suggest that unliganded VDR may promote adipogenesis and lipid accumulation in adipocytes (3), whereas Kong et al (13) suggest the opposite demonstrating an inhibitory effect of the unliganded VDR on adipogenesis. They further confirm their hypothesis by demonstrating that overexpression of hVDR completely blocks adipogenesis (13). They also suggest that 1,25-D binding to VDR stabilizes its levels which usually decrease few hours after induction of differentiation if unliganded and this is another possible mechanism for 1,25-D induced inhibition (13). These 2 studies have very clearly demonstrated an important regulatory role for 1,25-D in the adipose tissue biology. Figure 5, shows the potential molecular mechanism through which 1,25-D inhibits the differentiation process. +, means positively increase expression of the transcription factor. -, means downregulates or inhibits expression of the transcription factor. 1,25-D when introduced during induction of differentiation either blocks PPARγ activity directly or indirectly by blocking C/EBPβ which negatively regulates PPARγ. Another mechanism is 1,25-D binds

to VDRE (vitamin D response element) on the insig gene and increases Insig-2 (insulin-induced gene-2) expression. Insig-2 negatively regulates SREBP-1c and thereby blocks adipogenesis.

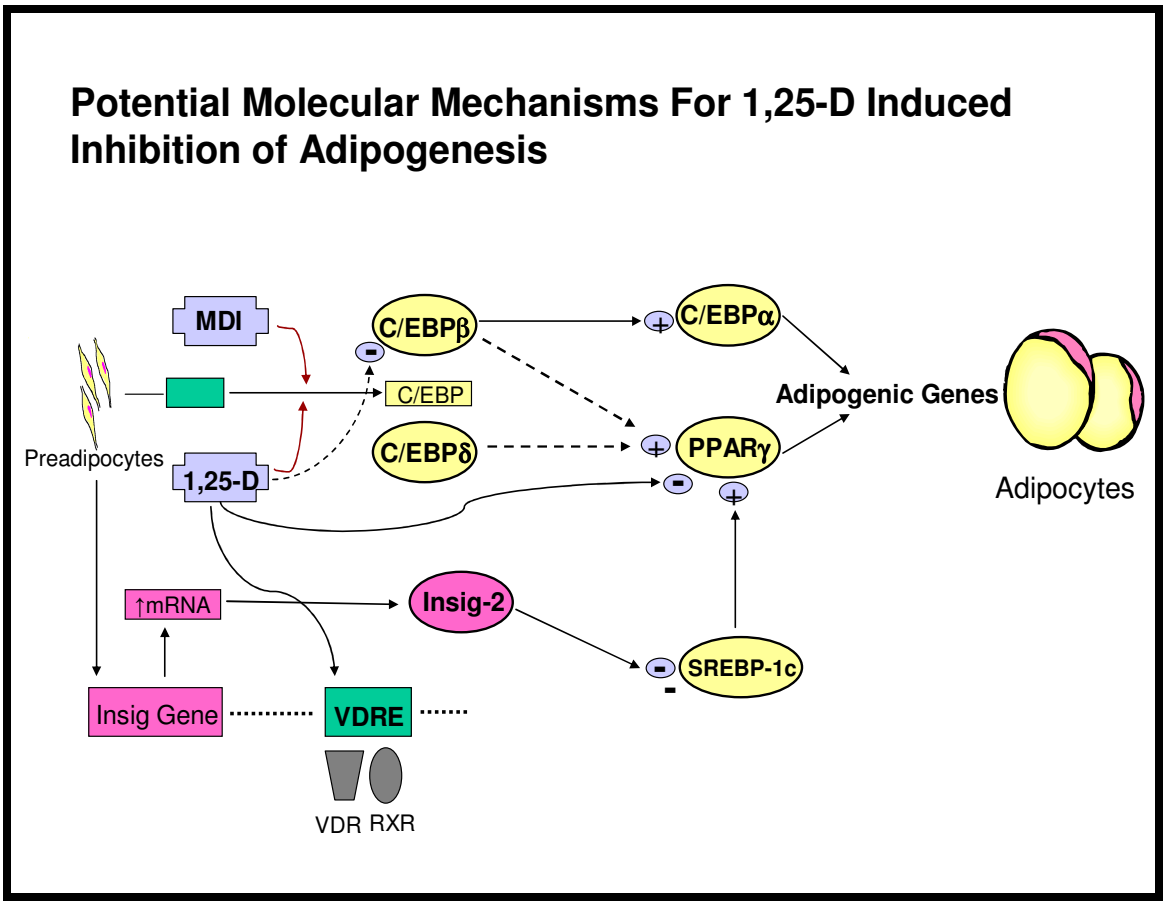
These effects seen *in vitro* have yet to be demonstrated *in vivo*. The expansion of the role of 1,25-D from its classical target organs like bone and intestine to other tissues like adipocyte, prostate etc has highlighted novel functions for this sunshine vitamin. It is of interest to examine the role of other vitamin D metabolites in these tissues. Serum concentration of 25-D is higher than any other vitamin D metabolite and it has the longest half life (3 weeks) (2). Furthermore, 1,25-D levels in the serum are in picomolar concentrations, have a very short half life and can be toxic at higher concentrations due to its hypercalcemic effect. It is therefore of interest to look at the role of 25-D in the differentiation process. The presence of the renal 1α-hydroxylase in extra renal tissues like prostate, placenta, breast and colon (16) elucidates a possible paracrine regulation for 1,25-D. To the best of our knowledge no studies have been conducted so far examining the role of 25-D on adipocyte differentiation. Thus studying the effect of 25-D on the differentiation process and the consequent expression of the enzyme 1α-hydroxylase in the adipocytes will highlight a possible local regulation of vitamin D within adipocytes.

Hence the hypothesis for the current research is:

(i) 25- D, the circulating form of vitamin D will inhibit the MDI-induced adipocyte differentiation of 3T3-L1 preadipocytes.

(ii) We also hypothesize that the inhibitory effects of 25-D will show decreased expression of the key adipogenic transcription factor PPARγ and the expression of 1α-hydroxylase enzyme in these cells.

Figure: 5. Potential molecular mechanism for 1,25-D induced inhibition of Adipogenesis. +, means positively increase expression of the transcription factor. -, means downregulates or inhibits expression of the transcription factor. 1,25-D when introduced during induction of differentiation either blocks PPARγ activity directly or indirectly by blocking C/EBPβ which negatively regulates PPARγ. Another mechanism is 1,25-D binds to VDRE (vitamin D response element) on the insig gene and increases Insig-2 (insulin-induced gene-2) expression. Insig-2 negatively regulates SREBP-1c and thereby blocks adipogenesis.



CHAPTER II

EXPERIMENTAL DESIGN / METHODS

A. Cell Culture and Differentiation

3T3-L1 cells (ATCC) were cultured in growth media constituting 89% Dulbecco's modified Eagle's medium (DMEM) obtained from Sigma-Aldrich, 10% calf serum (Sigma) and 1% penicillin/streptomycin (Gibco). The media was changed with fresh growth media every 48 hours. The cells were allowed to get 100% confluent. Two days post confluence differentiation was initiated. The 3T3-L1 cells was treated with differentiation media consisting of 0.5 mM 3-isobutyl-1-methylxanthine (MIX, Sigma), 1µM dexamethasone, 1.74 µM insulin (Sigma), 10% fetal bovine serum (Sigma), 1% penicillin/streptomycin and 87% DMEM. 1,25-dihydroxyvitamin D (Biomol Inc) was added at concentrations of 1 nmol/L, 10 nmol/L, 100 nmol/L and 0.1 nmol/L at the time of induction of differentiation. 25-hydroxyvitamin D was added at concentrations of 100 nmol/L, 250 nmol/L, 500 nmol/L, 750 nmol/L and 1000 nmol/L. The vehicle consisted of cells treated with 0.01% ethanol. The media was changed 48 hours post-induction to a media containing DMEM, fetal bovine serum, insulin and penicillin/streptomycin. Treatment compounds were added at same concentrations when media was changed. The extent of differentiation was assessed using Oil red O staining done on Day 6 of differentiation and through visual observations by the investigator under the microscope from day 1 through 6. The differentiation

experiments were conducted in 6-well plates. All treatment groups were in triplicates.

Cells were collected and harvested to assess mRNA of adipogenic genes PPARγ and for 1α-hydroxylase, VDR expression at on Day 6. All result analysis was done in triplicates.

A time course experiment was conducted at the lowest concentration showing inhibition for 1,25-D and 25-D treated cells to assess mRNA expression of 1α-hydroxylase, VDR for 1,25-D and 25-D at the lowest concentration which shows differentiation. The cells were collected and harvested for mRNA expression at day 0, 3h, 6h, day 1, day 2 and day 6 during differentiation,

B. Result Analysis

1. Oil Red O Staining

Oil Red O Stock: Oil Red O stock solution was prepared by mixing Oil red O powder (SIGMA Inc) in isopropanol and it is stirred overnight and filtered. This was stored at 4C.

Staining: The cells were collected on day 6 for oil red O staining. The media was discarded and 10% formalin was added briefly to the cells. The cells were washed with 60% isopropanol and completely dried. The cells were then stained with 60:40 Oil red O stock and isopropanol working solution and incubated in the Oil red O working solution for 10 min and washed 5 times with deionized water. The plates were completely dried and photographs were taken

using a digital camera. They were later eluted with 100% isopropyl alcohol and measured in a microplate reader at an OD 490nm. The results were expressed as mean of values from triplicate samples.

2. RNA Analysis

RNA Isolation:

The media was discarded and cells were harvested in TRI reagent (Invitrogen Inc) and scraped using a cell scraper. The RNA isolation was done as per instructions by the RNA kit supplier. After the phase separation using chloroform, the RNA was precipitated using 100% isopropanol and centrifuged. The supernatant was discarded and the RNA pellet was washed 3 times using 75% ethanol and solubilized in DEPC (RNA water). It was then stored at -80C until quantification.

RNA Quantification:

To measure RNA concentration a small quantity (4µg) of the RNA sample was combined in DEPC water and absorption was measured at OD of 260nm on a spectrophotometer.

Agarose Electrophoresis:

Gel was prepared by mixing 1.5% agarose and Tris acetate-EDTA (TAE) buffer. Agarose was dissolved making the solution clear. The loading dye was prepared and RNA samples were added and the gel was kept in the electrophoresis chamber for 30 minutes. Ethidium bromide (EtBr) was added and the plate was read under the illuminator and pictures were taken using a digital

camera. Agarose gel electrophoresis was run to check band integrity and quality before RT-PCR. The final mRNA expression after PCR running for gene of interest was also run on agarose gel electrophoresis.

RT-PCR:

Semiquantitative RT-PCR method was used to assess gene expression. The cDNAs were synthesized using SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen Inc) as per the protocol provided by manufacturer.

PCR of the first strand cDNA was performed using Platinum Blue PCR Supermix 96 kit (Invitrogen Inc). The cDNA was amplified for gene of interest using the respective primer sequence. The gene sequence for PPARγ primer were as follows: forward, 5'-CCA GAG TCT GCT GAT CTG CG-3' and reverse, 5'-GCC ACC TCT TTG CTC TGA TC-3'. PCR conditions for PPARγ were annealing temperature of 58C with 35 cycles. Sequence for the loading control primer GAPDH was forward, 5'- CAA GGT CAT CCA TGA CAA CTT TG-3' and reverse, 5'- GGC CAT CCA CAG TCT TCT GG – 3'. The PCR conditions for GAPDH was annealing temp at 60C and 40 cycles. The protocol used were as per provided by the manufacturer. The intensity of the PCR products were measured on agarose gel and pictures were taken using a digital camera. Band intensity was quantified using the NIH software ImageJ (Image Processing and Analysis in Java). Relative band intensity of PPARγ was calculated against the loading control GAPDH for all samples and the fold induction was calculated.

C. Statistical Analysis

The statistical tool used was SAS. The results were represented as mean ± SD. The ANOVA was used to assess the variability and Duncan t-test was used to assess statistical significance between treatment groups for the oil red o staining results. Scheffe's test was used for post hoc analysis and to control for experiment wise error. The statistical significance was assessed at p-value < 0.05. The treatment groups which exhibited large cell death were excluded from the analysis. MS Excel tool was used to calculate relative band intensity quantified using NIH software ImageJ for target genes of interest by dividing with the loading control GAPDH and fold induction for mRNA expression was measured.

CHAPTER III

RESULTS

A. 1,25-dihydroxyvitamin D inhibits differentiation of 3T3-L1 preadipocytes:

1,25-D was added to the differentiation media on day 0 which is the day of the initiation of differentiation. Oil red O staining was used to assess the degree of differentiation and was assessed on day 6 of differentiation. High color intensity suggests increased differentiation and triglyceride accumulation. Consistent with previous studies 1,25-D significantly inhibited differentiation at concentrations of 100 nmol/l, 10 nmol/l, 1 nmol/l and 0.1 nmol/l (3, 13). Figure 6 shows the oil red o staining pictures as observed on day 6. The cells treated with 1,25-D at 1 nmol/l and 0.1 nmol/l showed decreased color intensity as compared to the control suggesting decreased lipid accumulation and inhibition of differentiation. The degree of inhibition was highest in cells treated with100 nmol/l. We observed high cell death or apoptosis in adipocytes treated with 10 nmol/l and greater concentrations of 1,25-D. We did not further evaluate the cause for apoptosis as it was not within our research interest so for further experiments we used concentrations of 1,25-D where we did not observe cell death.

B. 25-hydroxyvitamin D inhibits differentiation of 3T3-L1 preadipocytes:

The prohormone 25-D was added on day 0 during initiation of differentiation at various concentrations and degree of differentiation was measured using Oil red O staining. 25-D also inhibited differentiation of 3T3-L1

cells at concentrations equal to or above 500 nmol/l. This inhibition was dose-dependent and significant at concentrations greater than or equal to 500 nmol/l. There was a small increase seen at 100 nmol/l and some inhibition seen at 250 nmol/l, although both were not significant. High cell death or apoptosis was observed in cells treated with 750 nmol/l and 1000 nmol/l of 25-D suggesting possible toxic concentrations. Similar to 1,25-D we used only those concentrations of 25-D which showed no cell death. Figure 7, shows the Oil red O staining pictures of cells treated with different concentrations of 25-D. The color intensity is lower in 25-D treatment groups as compared to the control (0.01% ethanol+MDI) which has highest red color intensity on day 6 suggesting high percent of differentiation. Inhibition is seen at 500 nmol/l, 750 nmol/l and 1000nmol/l 25-D treatment concentration. Figure 9 shows the summary of percent differentiation seen at various concentrations of 1,25-D and 25-D treatment as compared to the control (0.01% ethanol +MDI). The concentrations shown here are those where no cell death or toxicity was seen. The inhibition was statistically significant at 500 nmol/l for 25-D group and 1 nmol/l and 0.1 nmol/l for1,25-D concentrations. The values represent results from 4 independent experiments. There was no inhibition seen at 100 nmol/l 25-D.

We further evaluated the mRNA expression of PPARγ which is a key adipogenic gene at various concentrations of 25-D and 1,25-D. Studies have shown that it is the driving force for the differentiation process and decrease in its expression is consistent with inhibition of the adipogenic program (48). Figure 10, shows PPARγ and GAPDH mRNA expression at various concentrations of 25-D

and 1,25-D and the relative band intensity of PPARγ to GAPDH. It suggests a decrease in expression seen in cells treated with 1nmol/l showing a fold of 0.18 and a fold of 0.26 for 0.1nmol/l of 1,25-D after normalization for loading control GAPDH. PPARγ expression also is decreased at 25-D concentrations of 500nmol/l with a fold of 0.66 which is lower than the Day 0 control.

C. Time course evaluation of adipogenic gene PPARγ and vitamin D related genes:

Based on the results from the first phase of experiments we chose the least concentration at which significant inhibition of differentiation was seen in 1,25-D and 25-D to do a time course evaluation of expression of various vitamin D related genes during the differentiation process . We chose 500nmol/l for 25-D and 1nmol/l for 1,25-D. The time points used were 0h (day 0), 3h, 6h, 24h (day 1), 48h (day 2) and 144h (day 6). Inhibition of differentiation was seen as expected in 1,25-D and 25-D treatments on day 6. Figure 8 shows the Oil red O staining on day 6 for the treatment groups. Both 1nmol/l 1,25-D and 500nmol/l 25-D show a decreased red color intensity as compared to the control suggesting inhibition of differentiation. We further evaluated gene expression of the key adipogenic transcription factor PPARγ mRNA gene expression during the course of differentiation. The PPARγ gene expression in the control group shows a gradual increase in the mRNA expression during the course of differentiation. This is consistent with literature findings (3, 13). There was a decrease in gene expression of PPARγ mRNA on Day 6 seen in 1,25-D (0.1 nmol/l) and 25-D (500

nmol/l) treated cells. Figure 11 is the gene expression and fold change for PPARγ after normalization for the loading control GAPDH as seen on day 6. It shows a decrease in the amplicon of the bands of PPARγ in the 1,25-D (1 nmol/l) and 25-D (500 nmol/l) on day 6. The fold change for 1,25-D was lower than the day 6 control findings showing a 2 fold decrease as compared to the control and a similar decrease was seen in the 25-D group mRNA expression . This inhibitory effect of both 1,25-D and 25-D correlates with decreased PPARγ gene expression. However, as the sample size was n=1 more repeats need to be done to confirm these findings. Also consistent with previous studies PPARγ expression increases gradually during the process of differentiation (3, 13). Figure 11, also shows the increase in the PPARγ mRNA expression at 0, 3, 6, 12h, day 1, day 2 and day 6 in the control 1,25-D treated cells and in 25-D treated cells.

We evaluated the gene expression of vitamin D related genes including 1α-hydroxylase, VDR and 24-hydroxylase using real time RT-PCR conducted at Tufts University. The results suggest the presence of 1α-hydroxylase in 3T3-L1 cells. The expression of which occurs early during the differentiation process. Figure 12 shows the results from the real time RT-PCR for presence of 1α-hydroxylase at different time points in the control, 1,25-D and 25-D treated cells. The expression of which seems to peak at 6h and decreases as the differentiation process progresses suggesting that it is expressed early in the differentiation. 1α-hydroxylase expression appears to decline with initiation of differentiation. The 1α-hydroxylase expression in all 3 groups peaks at 6h point,

however the vitamin D metabolites show a smaller peak in the 1α-hydroxylase expression as compared to the control group. The VDR mRNA is also expressed early in the adipogenic process and decreases as the differentiation progresses. Figure13 shows that VDR expression in all 3 treatment group and it peaks at 6h in the control group. However the same peak is not seen in the 1,25-D and 25-D treated cells. This could be suggestive that the vitamin D metabolites appear to inhibit VDR mRNA expression. However VDR is expressed early in the adipogenic process in all 3 groups. The VDR results are consistent with previous studies conducted in this cell model (3, 13). These results are from experiments with a small sample size n=1. The enzyme 24-hydroxylase can be induced with the addition of both vitamin D metabolites. Figure 14, shows the 24-hydroxylase activity at various time points during differentiation with addition of vitamin D metabolites. It appears that 1,25-D as well as 25-D can induce 24-hydroxylase activity, however 1,25-D shows higher upregulation as compared to 25-D. This gene expression is highest at the 24h point in the 1,25-D treated cells. The 24-hydroxylase expression in 25-D treated cells was highest at the 6h point and decreases on day 1 (24h). The control did not show any 24-hydroxylase expression. This result needs to be repeated as the control data was missing for day 6. However it is important to note that the real time RT-PCR results of the vitamin D related genes are preliminary and have been conducted using a small sample size (n=1). These results need to be repeated on a larger sample. However there are some suggestive trends and consistencies in the key vitamin D related genes.

Figure: 6. Oil red O pictures of 1,25-D treated 3T3-L1 cells. 1,25-dihydroxyvitamin D treated 3T3-L1 cells assessed on day 6 inhibit the differentiation into mature adipocyte. Higher color intensity suggests increased differentiation. 3T3-L1 cells were cultured in GM- growth media; Con - control 0.01% ethanol+MDI and various concentrations of 1,25-D.

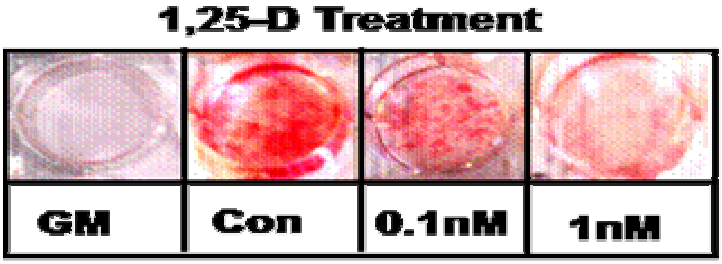


Figure: 7. Oil red O pictures of 25-D treated 3T3-L1 cells. 25-hydroxyvitamin D treated 3T3-L1 cells assessed on day 6 inhibit differentiation. Higher color intensity suggests increased differentiation or lipid accumulation. 3T3-L1 cells were cultured in GM- growth media; Con- control 0.01% ethanol+MDI and various concentrations of 25-D.

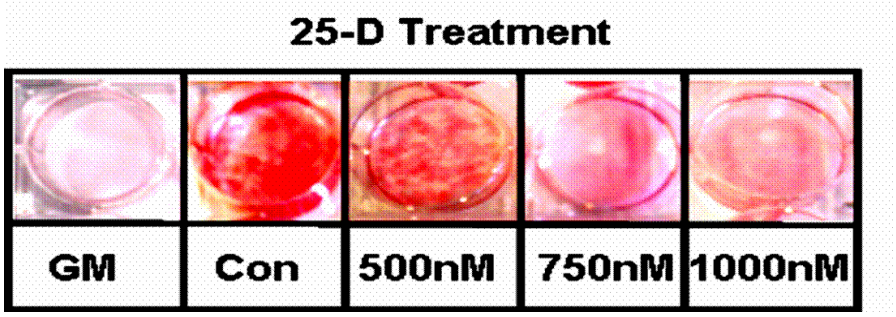


Figure: 8. Oil red O pictures of 3T3-L1 cells treated with vitamin D metabolites in Phase II. Results as seen on Day 6 of differentiation.

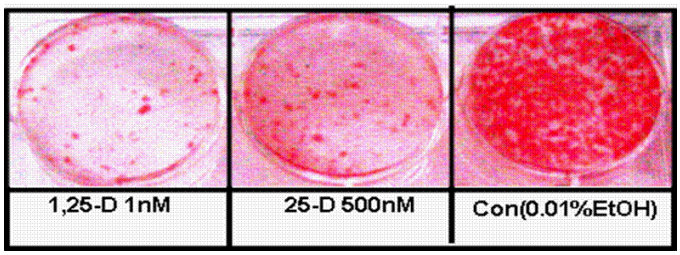


Figure: 9. The summary of results of Oil red O staining assessed on Day 6. The graph shows the percentage of differentiation seen in each treatment group against the control. The post hoc analysis by Scheffe's test for pairwise comparison was significant between the control and 25-D 500 nmol/l; 1,25-D 0.1 nmol/l and 1,25-D 1 nmol/l ($p<0.05$). The results are values from 4 independent experiments (n=4).

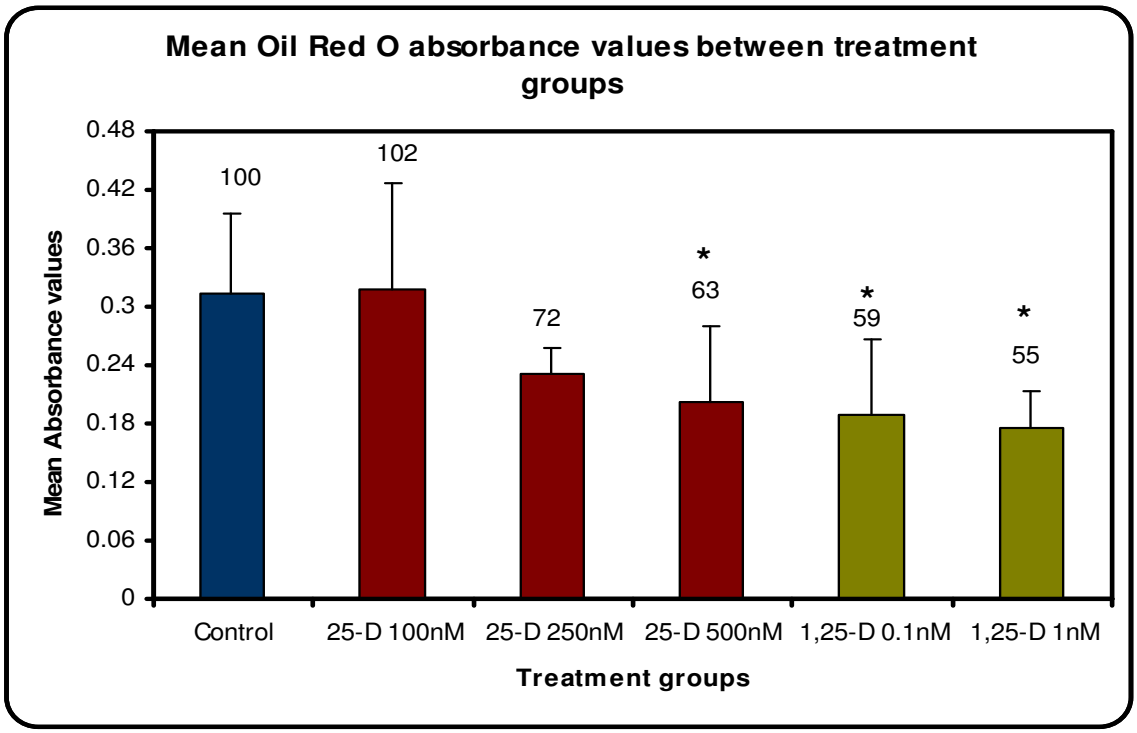
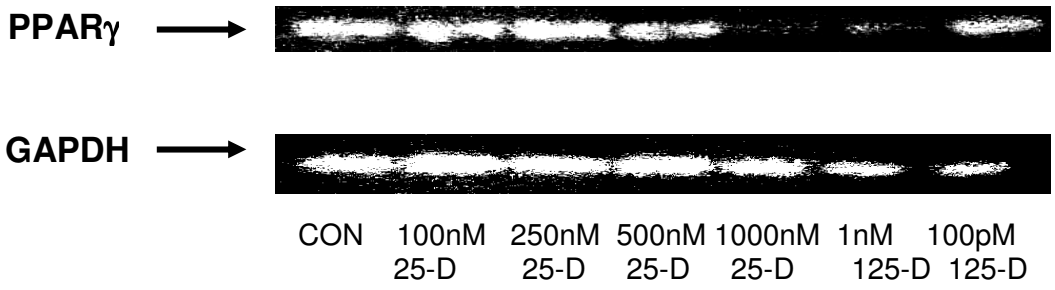


Figure: 10. PPARγ mRNA expression on Day 6 of differentiation at various concentrations of 1,25-D and 25-D. 3T3-L1 cells were harvested on day 6 and mRNA were isolated for various concentrations of 1,25-D and 25-D. Semiquantitative RT-PCR was performed for PPARγ and GAPDH and data was quantified after normalization for GAPDH (PPARγ/GAPDH). The results are representative of a sample size n=1.



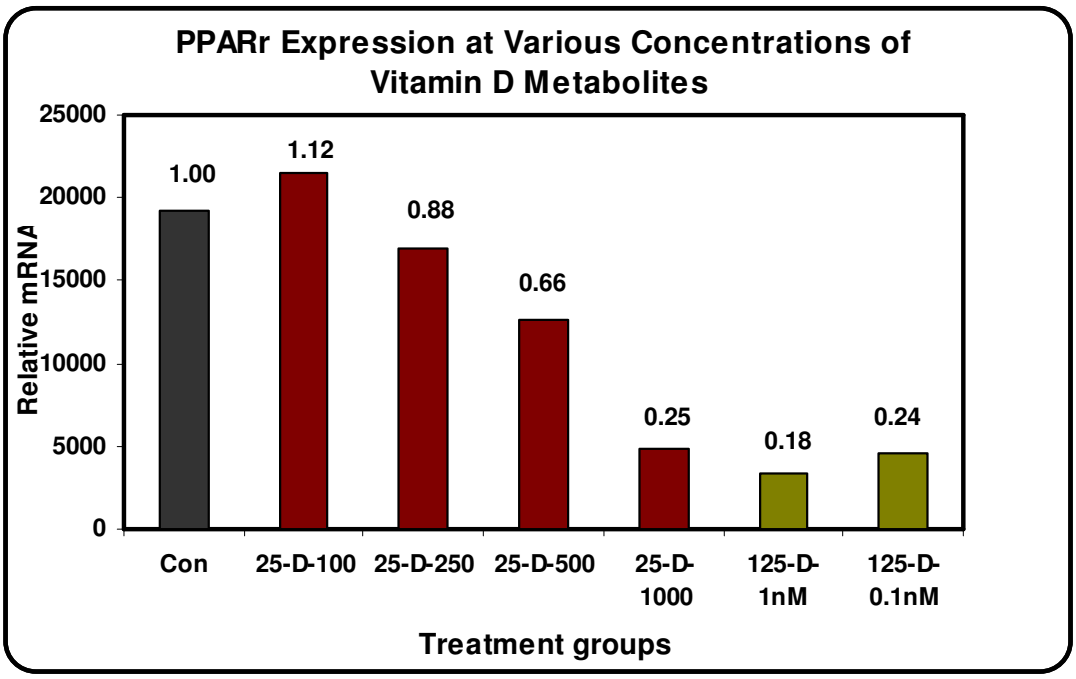
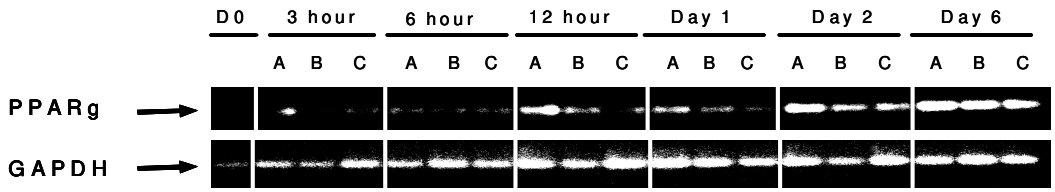


Figure: 11. PPARγ mRNA expression at various time points during the differentiation in all groups. 3T3-L1 cells were harvested on day 6 and mRNA were isolated for various concentrations of 1,25-D and 25-D. Semiquantitative RT-PCR was performed for PPARγ and GAPDH and data was quantified after normalization with the GAPDH mRNA (PPARγ/GAPDH). The results are representative of a sample size n=1.



D0 – Day 0
A; Control (0.01% EtOH)
B: 1,25 D (1nmol/l)
C: 25 D (500nmol/l)

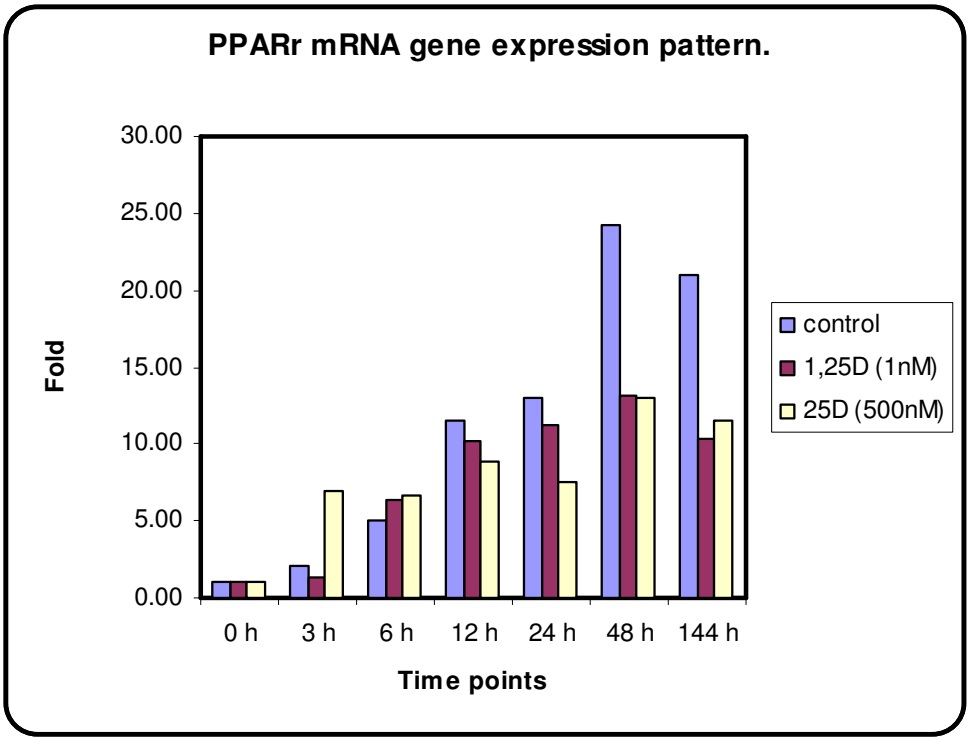


Figure: 12. Real Time RT-PCR time dependent expression of 1α-hydroxylase. Real Time RT-PCR results of 1α-hydroxylase mRNA gene expression during differentiation in all treatment groups. The data was normalized to the GAPDH mRNA for all samples. The graph shows the 1α-hydroxylase gene is expressed in the control, 1,25-D and 25-D treated 3T3-L1 preadipocytes at different time points during differentiation with a peak seen during the first few hours after differentiation. The data is representative of a sample size n=1.

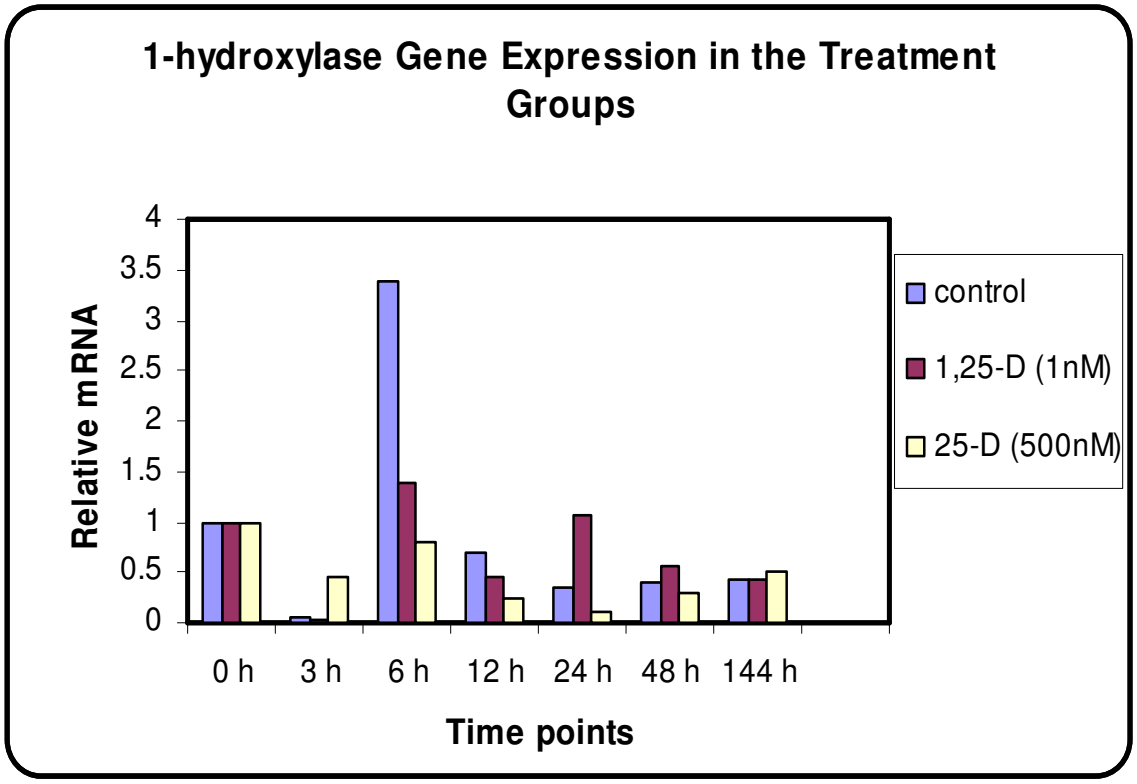


Figure: 13. Real Time RT-PCR time dependent expression of VDR. Real time RT-PCR was performed for VDR mRNA and the data was quantified after normalization for GAPDH expression. The graph shows that VDR gene is expressed in all the three treatment groups early during the differentiation process peaking at the 6hour time point in the control. The vitamin D metabolites appear to have decreased expression of this gene. The data is representative of a sample size n=1.

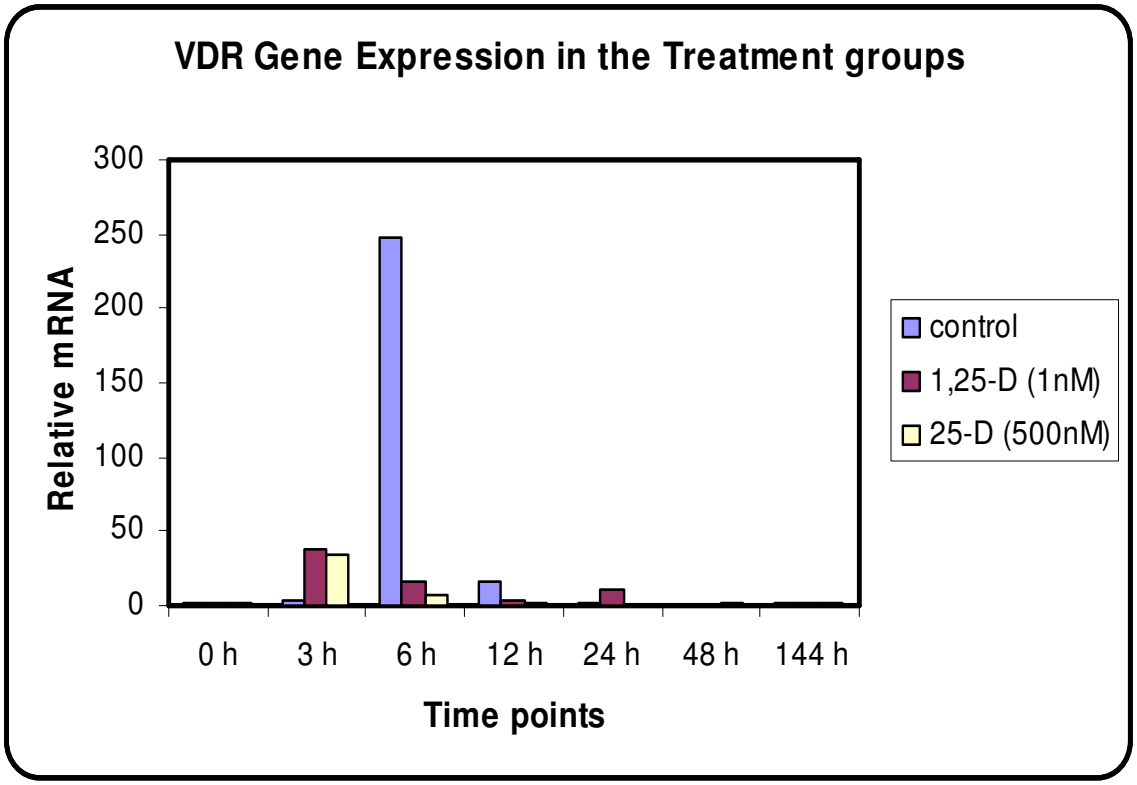
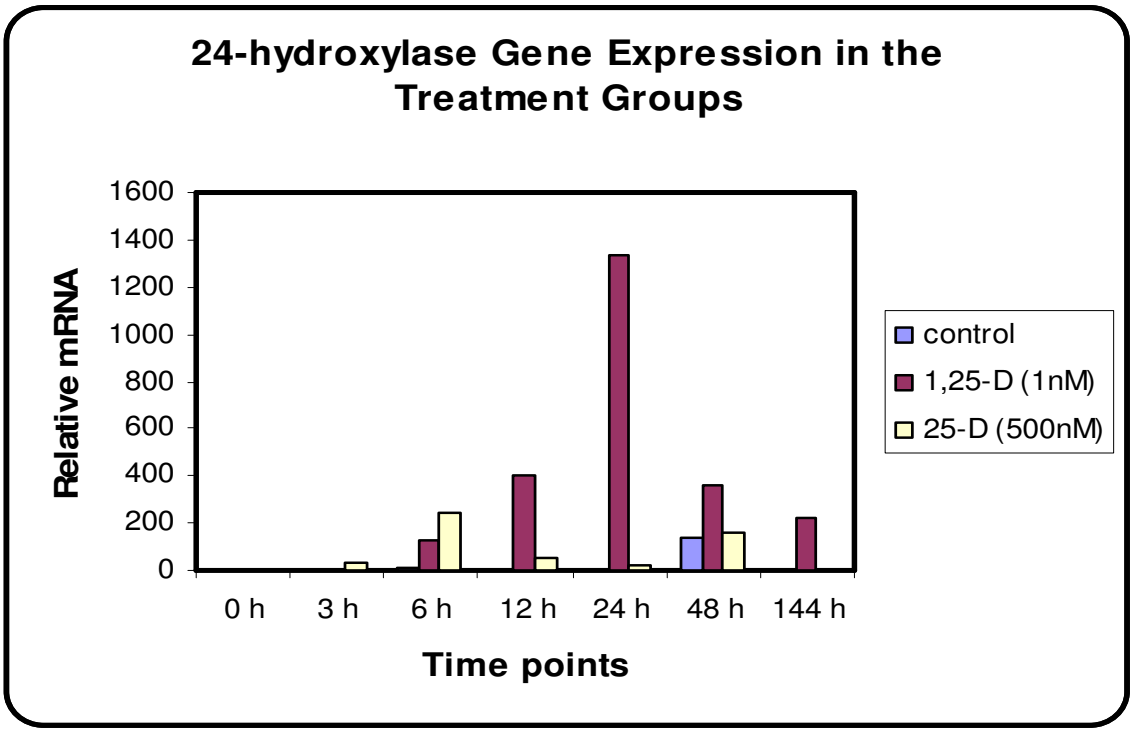


Figure: 14. Real Time RT-PCR time dependent expression of 24-hydroxylase. Real time RT-PCR was performed for 24-hydroxylase mRNA at various time points during differentiation and the data was quantified after normalization for GAPDH expression. The graph suggests that both vitamin D metabolites can induce 24-hydroxylase gene expression in 3T3-L1 cells. The gene expression peaks early during the course of differentiation and is highly induced by 1,25-D. The data is representative of a sample size n=1.



CHAPTER IV

DISCUSSION

The 3T3-L1 cell line is a good model to study the adipogenic program. Several studies have shown that 1,25-D inhibits adipogenesis (3, 10, 13, 24) and our study also demonstrated the inhibitory role of 1,25-D. However to the best of our knowledge no study has looked at the role of the prohormone 25-hydroxyvitamin D in adipogenesis and the current study attempts to understand its role and the gene expression of the key enzymes of vitamin D metabolism involved in this process. To investigate this role, we conducted a series of differentiation experiments with 25-D treatment using the 3T3-L1 cell model and evaluated the expression of important adipogenic and vitamin D related genes. 25-D is the prohormone metabolite of vitamin D. It is the most abundant form of vitamin D in the circulation. This prohormone can be converted to 1,25-D by the renal enzyme 1α-hydroxylase. 1,25-D is the active form of vitamin D and is responsible for the vitamin D related effects. Recent studies have identified the presence of the enzyme 1α-hydroxylase in extrarenal tissues such as breast, prostate, lung, placenta etc suggesting a possible paracrine regulation of vitamin D different from its traditional endocrine role (16, 54, 55, 56). This interesting find in other tissues was the basis for investigating the role of 25-D in the 3T3-L1 preadipocytes and if there is the presence of 1α-hydroxylase in these cells.

Our results demonstrate that 25-D can significantly inhibit differentiation of 3T3-L1 preadipocytes at concentrations equal to and greater than 500 nmol/l.

There is a clear difference in the color intensity by Oil red O staining between the control group and the 25-D treated cells. To demonstrate the validity of the methods used in our experiments we conducted the differentiation experiments with the addition of 1,25-D to the cells and we found an inhibitory effect similar to findings from previous studies (3, 10, 13). One *in vitro* study conducted by Rook et al (56) studied the effect of various vitamin D metabolites including 25-hydroxyvitamin D to control proliferation of M.tuberculosis in human monocytes. All metabolites inhibited the proliferation of M.tuberculosis and 25-hydroxyvitamin D caused inhibition at higher concentrations as compared to 1,25-D (56). Our results were similar in that 25-D caused inhibition of differentiation at higher concentrations as compared to 1,25-D. To further validate our Oil red O findings we studied the gene expression of PPARγ a key adipogenic transcription factor. It is an important factor promoting the development of the mature adipocyte from preadipocytes (19, 23). Studies suggest that several factors like C/EBPβ , SREBP-1c may activate PPARγ but no other transcription factor can promote adipogenesis in the absence of PPARγ (10, 19, 23). Similar to previous studies our results also demonstrated a decrease in PPARγ mRNA expression in the 1,25-D treated cells(3, 13) and a similar decrease in expression of PPARγ was seen in the 25-D treated cells. This is suggestive that the mechanism for the inhibitory effect seen in 25-D treated cells is also through the decreased expression of the key adipogenic transcription factor PPARγ.

We further evaluated gene expression of important vitamin D related genes like 1α-hydroxylase, VDR and 24-hydroxylase using real time RT-PCR

and observed promising trends. VDR and 1α-hydroxylase were expressed in the 3T3-L1 cells at various time points during the course of differentiation and 24-hydroxylase was induced by both vitamin D metabolites. In general, VDR gene was expressed early on during differentiation and decreased during the course of differentiation in all the groups. The VDR gene peaked between 3 and 6h after initiation of differentiation and decreased there on, consistent with results seen in studies by Blumberg et al and Kong et al studies (3, 13). However, we observed that the peak in VDR mRNA was highest in the control and both 1,25-D and 25-D treated cells showed smaller peaks. This suggests that the vitamin D metabolites may inhibit VDR expression. Previous studies have found that both PPARγ and VDR need to bind to retinoic acid receptor (RXR) for their action and they may compete with each other for RXR (3, 13). VDR binding to 1,25-D is required for 1,25-D to exert its effects (3,13). It is possible that RXR binds with the VDR early in the differentiation process makes it less available for PPARγ binding and hence causes the decrease in its expression in the 1,25-D and 25-D treated cells (13). Further, this decrease in PPARγ expression may cause the inhibition of differentiation seen with the addition of vitamin D metabolites. The early expression of VDR mRNA has been demonstrated by other studies and is suggested to be driven by the cAMP pathway. The same pathway is known to activate VDR in other cell types (3). Blumberg and colleagues (3) demonstrated that 3-isobutyl-1-methylxanthine which is a component of the MDI hormonal cocktail used to induce differentiation strongly induced VDR 6h after its addition.

This is consistent with the cAMP pathway driven induction of VDR and is the responsible for its early expression of the VDR mRNA (3).

In the present study 1α-hydroxylase mRNA was expressed in the 3T3-L1 cells. Similar to the VDR mRNA, 1α-hydroxylase expression appears to peak early during the differentiation process at the 6 hour point. The addition of the vitamin D metabolites after initiation of differentiation is associated with a decrease in the 1α-hydroxylase gene expression. However a peak in expression was noted at the same time point of 6 h in the cells treated with 1,25-D and 25-D similar to the control group. It is known that 1,25-D is a negative regulator of 1α-hydroxylase and upregulates 24-hydroxylase activity at an endocrine level in the body which could potentially explain a similar negative effect in its expression on addition of 1,25-D at the tissue specific level (27). It has also been suggested that the liganded VDR can repress the 1α-hydroxylase promoter. The liganded VDR binds to and blocks the activation function of VDIR (vitamin D interaction repressor), which binds and activates the 1α-hydroxylase promoter (3). 1,25-D needs to bind to VDR to exert its effects and that could be the mechanism by which the vitamin D metabolites are associated with decreased 1α-hydroxylase mRNA expression (3). Several studies conducted on cancer cell lines and monocytes have demonstrated that 1,25-D can upregulate 24-hydroxylase mRNA expression (54, 55, 57). The expression of 1α-hydroxylase, VDR, 24-hydroxylase in 3T3-L1 cells and the change in their levels seen on addition of 1,25-D or 25-D could be indicative of a tissue-specific role of vitamin D. Although the trends and consistencies seen in the key vitamin D related genes are very

promising, these experiments were conducted on a small sample size and hence caution must be exercised in interpreting these results and these observations need to be repeated on a larger sample size to confirm our findings.

Several *in vitro* cancer studies have showcased the antiproliferative and a tissue specific production of vitamin D (34, 54, 55). There have been studies conducted on breast tissues of normal and breast cancer patients that have demonstrated the expression of the key vitamin D enzymes with a higher expression in breast cancer tissues. These studies have highlighted a paracrine/autocrine role of vitamin D (54, 55). Another study which was conducted on monocytes showed inhibition of proliferation of M.tuberculosis caused by addition of 25-D. This study suggested the presence of 1α-hydroxylase in these cells which were capable of converting 25-D to its active form 1,25-D (56). The study demonstrated that radioactive 25-D got converted to a metabolite which cochromatographed with 1,25-D. They also demonstrated that more 25-D to 1,25-D conversion occurred with the addition of the lymphokine interferonγ (56). A similar role can be predicted for vitamin D in the adipose tissue; however more studies need to be conducted confirming our results on 3T3-L1 preadipocytes and on primary human preadipocytes. To the best of our knowledge our study is the first to demonstrate a role for 25-D in the adipocyte differentiation process in 3T3-L1 cells and the first to suggest the presence of 1α-hydroxylase in these cells.

In summary, our study has demonstrated that similar to 1,25-D the prohormone 25-D also inhibits the adipogenic process in 3T3-L1 cells. This

inhibitory effect correlates with the decrease in the expression of PPARγ which is a key adipogenic transcription factor. Preliminary findings suggest the presence of key vitamin D enzyme 1α-hydroxylase and other genes like VDR. 24-hydroxylase can be induced with addition of vitamin D metabolites. Both VDR and 1α-hydroxylase are expressed early in differentiation and inhibited with the addition of both 1,25-D and 25-D.

These results suggest that 25-D is biologically active in 3T3-L1 cells and its effects is suggestive of an intracellular conversion of 25-D to its active form 1,25-D in differentiating cells and a tissue specific effect of vitamin D in adipocytes. Further studies involving 25-D in the adipogenic process investigating the vitamin D related gene expression in this cell model as well as human preadipocytes will help elucidate a possible role for this prohormone 25-D in fat cell biology. With the rise in obesity and its associated comorbidities, the role of nutrients like vitamin D could hold clues to understanding the biology of fat cells and a possible role in obesity prevention.

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