The Suppressive Effects of 1,25-dihydroxyvitamin D3 and Vitamin D Receptor on Brown Adipocyte Differentiation and Mitochondrial Respiration

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DEDICATION

To my mother, Jennifer Fuller-Ricciardi, and my father, Charles Ricciardi, who have been my motivation, my support system, and my biggest fans.

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ABSTRACT

The vitamin D system plays a role in metabolism regulation. It has been reported that $1,25(OH)_2D_3$ [1,25-dihydroxyvitamin D] suppresses 3T3-L1 adipocyte differentiation. Vitamin D receptor (VDR) knockout mice showed increased energy expenditure whereas mice with adipose-specific VDR over expression showed decreased energy expenditure. Brown adipose tissue (BAT), which functions in non-shivering thermogenesis by uncoupling ATP synthesis from oxidation, plays important roles in energy expenditure. However, the effects of $1,25(OH)_2D_3$ on brown adipocyte differentiation and mitochondrial respiration have not been studied. Reported here is the mRNA expression of VDR, UCP1, and CYP27B1 (1α[alpha]-hydroxylase) in two mice models of obesity; and the down regulation of mRNA of VDR, CYP24A1 (24-hyrdoxylase), and CYP27B1 during brown adipocyte differentiation *in vitro*. $1,25(OH)_2D_3$ dose-dependently suppressed brown adipocyte differentiation, as revealed by oil red O (ORO) stained cell morphology, ORO absorbance, and brown adipocyte marker gene expression. Moreover, cellular bioenergetics measurements showed that $1,25(OH)_2D_3$ suppressed isoproterenol-stimulated oxygen consumption rates (OCR), maximal OCR and OCR from proton leak, but had no effects on ATP-generating OCR and spare respiration capacity in brown adipocytes. Consistently, over-expression of VDR also suppressed brown adipocyte differentiation. Furthermore, both $1,25(OH)_2D_3$ and VDR over expression suppressed PPARγ [gamma] transactivation in brown preadipocytes. Taken together, the results demonstrate the suppressive effects of $1,25(OH)_2D_3$/VDR signaling on brown adipocyte differentiation and mitochondrial respiration and suggest a role of $1,25(OH)_2D_3$/VDR signaling in regulating BAT function for obesity treatment and prevention.

Keywords: Vitamin D receptor, brown adipocyte, UCP1, mitochondrial respiration

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CHAPTER I
INTRODUCTION

Obesity, physiologically, is a result of hyperplasia and/or hypertrophy of lipid storing adipose tissue [1]. It is defined clinically as having a body mass index $\geq$30kg/m^2 and is currently a major health concern in the United States. In 2010, obesity surpassed smoking as the leading contributor to disease; which is not surprising considering the co-morbidities linked to excessive fat mass including: metabolic syndrome, Type 2 diabetes, heart disease, stroke, and cancer [2, 3]. Paralleling that statistic, the medical costs associated with obesity and its co-morbidities accumulated to an astonishing $147 billion dollars in 2008 [2]. With such a strong impact on the nation's health and economy, the exigency to resolve this problem is evident.

Humans possess two types of fat tissues: white adipose tissue and brown adipose tissue. White adipose tissue (WAT) is known for its lipid storing capacity and well-identified relationship with nutritional and appetite markers and adipokines such as glucose, leptin, ghrelin, and insulin. Moreover, white adipose tissue participates systemic inflammation through the secretion of chemokines and cytokines.

Brown adipose tissue (BAT) functions to produce heat through non-shivering thermogenesis. The presence of brown adipose tissue in small eutherian mammals, such as mice, provides adequate thermoregulation in a cold environment without compensatory shivering [4]. BAT's thermogenic capacity is conferred by the actions BAT specific uncoupling protein 1 (UCP1). UCP1, located in the inner mitochondrial membrane of brown adipocytes, uncouples ATP synthesis from respiration, resulting in dissipation of heat [5, 6]. Until recently, human BAT was thought to exist exclusively in neonates and that maturation, resulting in fat deposition and skeletal muscle development, lead to BAT regression. However, positron emission

tomography/computed tomography scanning of adult humans has revealed the presence of fat depots in the cervical, supraclavicular and paravertebral regions with the characteristics of BAT [7-11]. Furthermore, recent gene expression profiling of adult humans has confirmed many classical BAT features in the fat depots of the supraclavicular regions [12, 13]. Due to its energy expending activity, BAT has become the novel target tissue for combating obesity [14-19]. It has been discovered that BAT is negatively associated with BMI, visceral fat, visceral/total fat, waist circumference, and diabetes [20, 21].

Accumulating evidence suggests that vitamin D has pleiotropic actions that can affect multiple organs and metabolic processes, in addition to maintaining calcium homeostasis and skeletal health. Recent studies have suggested that WAT is a direct target of vitamin D, and that the hormone modulates adipose tissue formation and function [22]. The vitamin D system has been implicated in white adipose tissue in terms of inflammation, glucose regulation, adipogenesis, and energy metabolism [23-26]. Likewise, the expression of VDR has been reported in murine 3T3-L1 preadipocytes [27, 28] and human preadipocytes [29].

It has also been reported that 1, 25$(OH)_2D_3$ inhibits 3T3-L1 adipocyte differentiation [27, 28], but promotes adipocyte differentiation from human subcutaneous preadipocytes [29]. Further, studies of VDR knockout mice and transgenic mice with adipose-specific VDR over-expression revealed that energy expenditure was markedly higher in VDR knockout mice, but reduced in the VDR transgenic mice, compared to their respective controls, suggesting a role of the vitamin D system in energy metabolism [30, 31].

BAT plays a critical role in energy expenditure; however, whether vitamin D system modulates brown adipose tissue formation and function has not been studied. The objective of the thesis research is to fill in the knowledge gap regarding the effects of vitamin D, specifically

vitamin D_3, and the vitamin D receptor on brown adipocyte differentiation. By employing a brown preadipocyte cell line, the effects of $1,25(OH)_2D_3$ and VDR expression on brown adipocyte differentiation were studied. The results presented in this thesis add to our understanding of the nutritional regulation of brown adipocyte development and function.

CHAPTER II
LITERATURE REVIEW

2.1 Overview of Vitamin D

2.1.1 Vitamin D Structure and Function

Despite being defined as a vitamin, vitamin D is truly a secosteroid hormone. As a hormone, vitamin D has a number of calceamic and noncalceamic biological roles. Its classical role is associated with bone metabolism as it is required for adequate enterocyte absorption of calcium by increasing the expression of calcium channels for uptake in the intestine. Likewise, it acts within the kidney to promote calcium reabsorption in the distal tubule via activation of parathyroid hormone [32]. Moreover, it also aids in the absorption of phosphorus, an essential mineral in bone maintenance [33, 34]. Recently, vitamin D has been recognized in the scientific community as an immunological, anti-inflammatory, and transcription-modulating molecule. In either mice or human trials, vitamin D has been linked to diminishing the effects or reducing the risk of various inflammatory states such as non-obese type 1 diabetes, multiple sclerosis, lupus, and inflammatory bowel disease [35-38]. Because of its diversity in human health, it is no surprise that serum vitamin D deficiency has been associated with early mortality, myocardial infarction, diabetes, various cancers, and obesity [39-42].

The binding of vitamin D_3 to the vitamin D (VDR) receptor is necessary to produce the aforementioned biological effects. The VDR has a high affinity and specificity for the bioactive $1,25(OH)_2D_3$ ligand. The binding of the ligand and the receptor is required for action mediation in a variety of human tissues including osteoblasts, macrophages, pancreatic beta cells, smooth muscle, adipocytes, and epithelial cells [22]. Vitamin D_3, also identified as $1,25(OH)_2D_3$, is

understood to modulate gene expression via two mechanisms: the first being interaction of transcriptional promoter genes and the retinoid X receptor/VDR heterodimer and the second being posttranslational modifications from calcium flux induction. The former mechanism begins with the introduction of free $1,25(OH)_2D_3$ to the cell. Free $1,25(OH)_2D_3$ binds to the VDRs, which then become phosphorylated, resulting in a significant conformational change that closes the receptor, thereby activating it. Phosphorylated vitamin D receptors bind to retinoid X receptors and create a heterodimer complex that is able to activate or repress transcription at promoter genes after stabilization on DNA vitamin D response elements [43]. VDR mRNA expression has been identified in most human tissues, including osteoblasts, keratinocytes, smooth muscle, epithelial cells and white adipocytes, supporting the diverse effects of $1,25(OH)_2D_3$ signaling.

One example of a non-classical role of vitamin D is in the immune system. It is understood that numerous adaptive and innate immunity cells express the vitamin's receptor. Moreover, both macrophages and dendritic cells of the immune system posses the 1α-hydroxylase enzyme, resulting in hydroxylation of $25(OH)D_3$ to yield bioactive $1,25(OH)_2D_3$. When pattern recognition cells sense a pathogen, they signal for increased expression of both the vitamin D activating enzyme and VDR. Ligand binding elicits an immune response via transcriptional activation and/or repression of the target genes of the downstream signaling pathways [32].

There is also evidence to support the activity of the vitamin D/VDR complex in the vascular system. Similar to the immune system, both VDR and 1α-hyrdoxylase have been identified in cardiac myocytes, fibroblasts, and smooth muscles cells. Vitamin D deficiency in humans has been associated with congestive heart failure, peripheral vascular disease, and

coronary artery disease. Similarly, in mice, vitamin D deficiency and VDR deletion is associated with elevated blood pressure and hyperreninemic hypertension with cardiac hypertrophy, respectively [32]. In a rodent study, rats were infused with 800 ng/kg/min of angiotensin II for a 14-day period. This resulted in elevated blood pressure, elevated cardiac interstitial fibrosis, myocyte hypertrophy, and increased expression of the hypertrophy-sensitive fetal gene program. Interestingly, concurrent treatment with $1,25(OH)_2D_3$ analog, paricalcitol, ameliorated these cardiac effects [44].

These are just a few examples of the expansive involvement of both the vitamin D ligand and VDR in mediating human biology. The involvement of the vitamin D complex in adipose tissue will be further elucidated later.

2.1.2 Dietary and Non-dietary Sources of Vitamin D

Vitamin D can be found in a variety of animal products including liver, eggs, milk, cheese, mushrooms, and some saltwater fish. The American food supply is also fortified with vitamin D. Fortified products include: orange juice, yogurt, and some cereals. Additionally, vitamin D is created endogenously. Cholecalciferol can be synthesized in the skin and transformed to bioactive calcitriol through a series of biological processes [33].

2.1.3 Vitamin D Metabolism and Transportation

Out of the two forms of vitamin D, vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol), vitamin D_3 is the only form that is found naturally in humans and animals due to the synthesis of vitamin D_3 from 7-dehydrocholesterol in the skin upon ultraviolet B irradiation. When 7-dehydrocholesterol in skin is exposed to UVB it is transformed into

previtamin D3. Further structural transformations produce vitamin D_3 (cholecalciferol), allowing for translocation from the skin to the blood stream. Upon admission into the blood stream, vitamin D_3 is bound to α1-globulin, a vitamin D binding protein. Once cholecalciferol reaches the liver, it is hydroxylated by 25 hydroxylase (encoded by CYP27A1) to release 25-hydroxycholecalciferol ($25(OH)D_3$) into circulation. In the kidney, $25(OH)D_3$ undergoes further hydroxylation by 1α hydroxylase (encoded by CYP271B) to complete the conversion of cholecalciferol to bioactive calcitriol ($1,25(OH)_2D_3$) [33]. A variety of cytochrome P450 enzymes, including 24-hydoxylase (encoded by CYP24A1), initiate the inactivation and degradation of vitamin D_3 and its metabolites by forming calcitrioic acid [33, 45]. A visual schematic has been provided to illustrate the metabolism of vitamin D (Fig. 1).

The genomic action of $1,25(OH)_2D_3$ is mediated through the vitamin D receptor (VDR), which upon binding to $1,25(OH)_2D_3$ forms heterodimer with retinoid X receptor (RXR) and binds with vitamin D_3 response elements in the promoters of target genes to regulate the target genes transcription.

2.1.4 Vitamin D and Energy Metabolism

The effects of vitamin D on energy metabolism have been studied to some extent. The most pertinent research regarding the current hypothesis assesses the impact of vitamin D and the VDR complex on white adipocyte differentiation, function, and total cellular metabolism.

There is evidentiary support for inhibition of 3T3-L1 cell differentiation by $1,25(OH)_2D_3$ [22, 46]. When treated with calcitriol, mRNA and protein expression of adipocyte marker gene PPARγ, C/EBPβ, and SREBP1 are dose-dependently down-regulated. Likewise, $1,25(OH)_2D_3$ has been reported to impair lipogenesis in preadipocyte cells by promoting expression of insulin

induced gene 2 (Insig2), a gene encoded to block fatty acid synthesis by modulating the proteolytic activation of sterol regulatory element-binding proteins [47]. Moreover, up regulation of the VDR in the absence of calcitriol has been reported to inhibit adipocyte differentiation [22]. While these results paint a convincing picture of the suppressive effects $1,25(OH)_2D_3$ on adipocyte differentiation of murine derived 3T3-L1 [27, 28], it has been reported that a similar dose of $1,25(OH)_2D_3$ (i.e., 10^{-8} mM or 10 nM) actually *promoted* differentiation of *human* subcutaneous preadipocytes [29].

In characterizing the molecular mechanisms by which vitamin D_3/VDR suppresses 3T3-L1 adipocyte differentiation, it was found that VDR mRNA expression reached a maximum 6 hours after induction of white adipocyte differentiation [46]. Moreover, VDR nuclear proteins imperative for VDR function, showed exponential accumulation at 4 hours and reached maximum expression at 12 hours post induction [46]. These results suggest that the vitamin D system is active at the early stages of adipocyte differentiation [46, 48].

The difference between liganded and unliganded VDR in hindrance of normal adipogenesis has also been noted [46]. The VDR siRNA transfected cells without calcitriol did not significantly reduce PPARγ [46] . However, in the presence of calcitriol, expression of PPARγ and C/EBPα, an essential transcription factor that works in concert with PPARγ in adipogenesis, were reduced in 3T3-L1 cells over expressing VDR, likely due to the blocking of the C/EBPβ pathway [46, 49]. Thus, liganded VDR seems to be the most potent inhibitor of white adipocyte differentiation by limiting lipid accumulation and diminishing the production of PPARγ and C/EBPα [22, 46, 49].

Additionally, the effects of vitamin D on thermogenesis of white adipocytes were reported [50]. With as low as 1 nM vitamin D, both basal and pharmacologically induced expression of uncoupling protein 2 (UCP2) were diminished whereas a specific agonist for the membrane vitamin D receptor (mVDR), 1α,25 dihydroxylumisterol 3 was unable to show the same effects. Nuclear VDR knockdown blocked the inhibition by $1,25(OH)_2D_3$ whereas a specific mVDR antagonist, 1α,25-dihydroxyvitaminD_3, was unable to prevent the $1,25(OH)_2D_3$ inhibition of UCP2 expression [51]. These results suggest that thermogenesis of WAT is negatively impacted by the vitamin D/VDR complex through its genomic action [51].

To further study the role of the VDR as it relates to adipogenesis and metabolism *in vivo*, an aP2-hVDR transgenic mouse model was created to specifically over express hVDR in adipocytes [52]. It was found that mice over expressing hVDR had a greater body fat percentage than their wild type counterparts on the standard chow diet [52]. Likewise, transgenic mice on the high fat diet had a more significant weight gain than wild type mice on the high fat diet. Interestingly, transgenic mice exhibited elevated cholesterol compared to the controls but they did not have statistically significant non-esterified fatty acids or triglycerides [52]. Metabolism was also altered in the transgenic mice. Expression of carnitine palmitoyl transferase 1 and 2, hexokinase, pyruvate kinase, hormone sensitive lipase, adipose triglyceride lipase, and uncoupling protein were reduced in adipose tissue of transgenic mice, leading to impaired β-oxidation, glucose metabolism, and lipolysis [52].

To further illuminate the effects of VDR *in vivo*, literary evidence suggesting that VDR null mice maintain a leaner phenotype is described [26]. Ablation of the VDR *in vivo* was associated with a lower body fat in male and female mice on a high fat diet. Interestingly, BAT in VDR null mice was less abundant than in wild type regardless of the diet. However, null mice

were able to maintain better BAT cell morphology i.e., shape and appearance, with fewer lipid droplets than the wild type counterparts. On the high fat diet, leptin levels were decreased in VDR null mice. Moreover, oxygen consumption rates, CO_2 production, and total energy expenditure were significantly higher in VDR(-/-) mice without significant changes in dietary intake or physical activity [26]. Together, these results suggest that VDR is a negative regulator of adipose energy expenditure.

Yet another study demonstrated that VDR knockdown can modulate energy metabolism *in vivo* [53]. This particular research supports the idea that the VDR/vitamin D ligand is a key regulator in adipogenesis and glucose metabolism. These researchers compared body weight, abdominal WAT, leptin, and food intake in young VDR knockout mice and wild type mice on a C57BL6 background fed with a rescue diet. It was found that VDRKO mice weighed significantly less at 2, 4, and 6 months of age compared to their age matched wild type counterparts. They also found almost 50% more abdominal white fat in the wild type mice at the 6th month. Not surprisingly at the 4 and 6 month check points, adipokine leptin was significantly reduced in mice lacking VDR. The food intake, measured in grams per day, was notably indifferent at the 2 and 4 month time points. Unexpectedly, VDRKO were actually consuming more food (g/day) at 6 months. To examine the effects of VDR ablation on interscapular BAT (iBAT), interscapular brown fat was isolated and weighed and no significant difference in iBAT mass was found.

This same group of researchers chose to further research the effects of VDR elimination in a different strain of mice genetically prone to obesity and impaired glucose metabolism: CD1 mice [53]. When comparing the CD1 WT to the CD1 VDRKO, CD1 VDRKO mice weighed less and had significantly less abdominal and subcutaneous fat mass. This held true even when the

dietary fat was increased from 5% to 15%. Interestingly, unlike in the C57BL6 model, CD1 mice did show differences in iBAT mass. On both 5% and 15% dietary fat diets CD1 WT mice had larger amounts of iBAT than the CD1 VDRKO mice despite CD1 VDRKO mice consuming significantly more calories than their WT counter parts [53].

When comparing the metabolic parameters including serum glucose, insulin, and triglycerides, not surprisingly, the CD1 mice fed a 5% fat diet showed higher glucose, insulin and triglycerides than the C57BL6 mice. However, in both CD1 and C57BL6 VDRKO models, glucose, insulin, and triglycerides were normal. This suggests that even in cases of genetic obesity, VDRKO can protect against metabolic abnormalities [53].

The next question raised is whether or not CYP27B1 ablation, the enzyme required for $1,25(OH)_2D_3$ synthesis, produces any remarkable changes. On both the 5% and 15% fat rescue diets, CYP27B1 KO mice were lighter than the wild types with smaller abdominal adipose tissue stores. Like the VDRKO mice, CYP27B1KO mice were hypoleptinemic, hyperphagic, and had exhibited no differences in iBAT [53].

To elucidate the mechanism by VDR modulates the lean phenotype, researchers measured mRNA expression of PPARγ and fatty acid synthase (FAS) and found that neither PPARγ nor FAS were significantly different between knockout and wild type mice. In contrast, they noted that UCP1 mRNA was 25 fold higher in the WAT of VDRKO than in that of wild type mice. These results suggest that VDR may negatively modulate energy expenditure through suppression of UCP1 in WAT, leading to a lean phenotype in VDR null mice [53].

Taken together, these studies suggest the significant impact of vitamin D and/or VDR on the development of BAT and WAT and whole body energy metabolism.

2.2 Brown Adipose Tissue: Significance and Nutritional Regulation

2.2.1 Brown Adipose Tissue Function

Brown adipose tissue (BAT) has been identified as a highly vascularized thermogenic tissue responsible for non-shivering, adaptive thermogenesis during cold exposure [54, 55]. It is a multilocular tissue because its cells are composed of many small lipid droplets dispersed in the cytosol surrounding a central nucleus. It also contains a high number of mitochondria, the organelle responsible for the generation of heat that occurs in these cells. In contrast, WAT is unilocular with a singular lipid droplet, few mitochondria, and peripheral nucleus. White adipose tissue is much less metabolically active than BAT because its major function is triglyceride storage [56].

Brown adipose tissue's thermogenic capacity is conferred by its expression of UCP1, which is confined to the inner mitochondrial membrane. Under activation of the sympathetic nervous system UCP1 enhances energy expenditure by uncoupling oxidative phosphorylation from ATP synthesis [55]. Normally, the process of the electron transport chain's (ETC) oxidative phosphorylation produces free energy that drives adenosine triphosphate (ATP) synthesis. The proton gradient produced by the oxidation-reduction reactions of the ETC is utilized by ATP synthase converting adenosine diphosphate (ADP) to ATP. Therefore, the respiratory capacity of a cell is limited by the efficiency of mitochondrial phosphorylation of ADP to ATP. In brown adipocytes, the transmembrane protein UCP1 creates a proton leak, uncoupling these processes and consequently, generates heat. This process allows for continuation of fatty acid oxidation with a low rate of ATP synthesis [57, 58]. It has also been suggested that glucose is more highly

metabolized via anaerobic glycolysis in BAT to compensate for the impaired ATP synthesis of the ETC [58].

Previously, BAT was believed to be present in infants only. Because infants have minimal skeletal muscle for shivering, BAT was presumed to be the mechanism by which infants maintained temperature homeostasis. Approximately 2-4% of the birth weight can be attributed to BAT. However, at about one month after birth, brown adipose tissue is significantly reduced [59]. BAT degeneration is a result of apoptosis induced by tumor necrosis factor alpha [60]. With the use of Positron Emission Tomography associated with Computed Tomography (PET-CT), researchers have located depots of BAT in adults [7-11, 61]. Radioactive glucose analogue ^{18}F-flurodeoxyglucose (FDG) is traditionally used to identify tissues with glycolytic activity in PET scans. Intravenous administration of the analogue unintentionally discovered depots of BAT in adult humans due to its unsuspected uptake of FDG [62]. Currently, FDG uptake is the gold standard in measuring BAT [55]. The largest depots of this metabolically active tissue can be found in the: supraclavicular, suprarenal, paravertebral (interscapular), and neck regions [21, 61, 63]. Statistical analyses have indicated that, in humans, BAT is negatively correlated with outdoor temperature and age. Furthermore, women have greater BAT mass and glucose uptake than men; though, this disparity between the two genders seems to diminish as age increases [21].

2.2.2 Brown Adipose Development

In white adipogenesis, cellular differentiation begins with rapid expression of transcription factors CCAAT/enhancer-binding protein β (C/EBPβ) followed by C/EBPα, PPARγ, and sterol regulatory element-binding protein 1 (SREBP1). The upregulation of these

proteins results in the development of an adipocyte phenotype, as evidence by adipogenic enzyme markers such as lipoprotein lipase and fatty acid synthase [22]. It has been reported that in normal 3T3-L1 differentiation, VDR is also expressed in the early stages of differentiation and is maintained in the presence of $1,25(OH)_2D_3$ [22].

Classical brown adipocytes are derived from Myf5-positive myoblastic cells [58, 60]. The development of brown adipocytes is controlled largely by transcription factor PPARγ and transcriptional coactivator, PPARγ coactivator 1α (PGC1α).

PGC1α is responsible for the promotion of UCP1 expression by interacting with PPARγ and RXR heterodimer, allowing for transcription of genes involved in mitochondrial oxidative phosphorylation and UCP1 expression. Likewise, mitochondrial biogenesis relies on PGC1α for nuclear respiratory factor induction (Nrf-1). Nrf-1 is responsible for inducing the expression of the genes of the electron-transport chain. Not surprisingly, PGC1α is expressed at higher levels in BAT than in WAT and is up regulated following cold exposure and β adrenergic stimulation via the p38 MAPK pathway [64].

When ectopic expression of PGC1α is permitted in WAT, they adopt a phenotype similar to brown adipocytes, expressing UCP1 [19, 65]. Moreover, fatty acid metabolism enzymes in WAT increase with PGC1α expression, suggesting the potential for advanced fatty acid metabolism in browning white adipocytes [65]. Some research suggests the necessary involvement of zinc finger containing protein PR domain containing 16 (PRDM16) in conjunction with PGC1α, PPARγ, and C/EBPβ in the browning of white preadipocytes via promotion of thermogenic and brown adipogenic gene expression [16, 17, 66].

PRDM16 is involved in both BAT and WAT development by simultaneously inhibiting the expression of WAT genes via transcriptional corepressors and promoting the expression of BAT genes through interaction with PGC1α [67, 68]. BAT gene expression is enhanced by PPARγ stabilization of PRDM16 on the promoter regions of BAT specific genes [17, 67, 69].

A number of cell types have been identified as having potential to adopt a brown phenotype, contributing to uncoupling and thermogenesis. There is some evidence to suggest that human subcutaneous tissue contains a distinctly different beige adipocyte precursor cells that are identified by expression of genes CITED1 and CD137 [70]. While these cells express typical WAT genes, they have a unique capacity to also express UCP1. These cells, when appropriately stimulated with PPARγ agonists, can differentiate into functional beige or brown adipocytes. Interestingly, subcutaneous adipose tissue samples from obese individuals had impaired UCP1 gene and protein expression when compared to the lean individuals. These data suggest incapacity of BAT in obese individuals to function maximally [70].

Human multipotent adipose-derived stem cells (hMADs), with prolonged exposure to PPARγ, are able to differentiate into functional brown adipocytes, characterized by their expression of UCP1 and Cidea [71]. Thyroid hormone triiodothyronine may also have the capacity to promote the browning of hMADS. In one study, the introduction of triiodothyronine to hMADS resulted in elevated expression of the following thermogenic genes: UCP1, PGC1α, NRF1, TFAM, Cidea, and Elovl [72]. Subsequently, oxygen consumption rates were significantly increased in triiodothyronine treated groups, suggesting enhanced mitochondrial biogenesis [72].

Muscle derived CD34+ cells are another potential progenitor cell with the ability to

express a brown adipocyte phenotype [73]. One study utilized tissues extrapolated from humans (adult and fetal) as well as mice for examination. A variety of skeletal muscle cells were identified during the course of this study; interestingly, the CD34+ cells of expanded fetal muscles showed gene expression similar to that of brown adipocytes. Upon further investigation with RT-PCR, it was found that differentiated skeletal muscles expressing the CD34+ protein on the cell surface demonstrated UCP1, PPARγ, PGC1α, β_3-adrenoreceptor, and Cidea mRNA expression. A second finding of this study was that CD34+ cells' UCP1 expression could be up regulated by exposure to pharmacological treatment. UCP1 mRNA was increased approximately seven to eightfold by cAMP derivatives in both primary culture and expanded cells. Interestingly, PPARγ agonist rosiglitzone proved to create an eightfold increase in expanded cells but yielded no statistically significant change in primary culture cells. The reason for this incongruity is unknown. These findings suggest that CD34+ cells in fetal muscle tissues have the capacity to express UCP1 and develop into functional brown adipocytes provided proper stimulation [73].

Lastly, adipocyte-like skin dermal cells from individuals with Hereditary Vitamin D Resistant Rickets (HVDRR) have been examined for their capacity to brown [74]. In this particular study, UCP1 expression was increased in the skin dermal cells from HVDRR individuals when compared to the controls [75]. The study also revealed that the suppression of UCP1 mRNA by VDR appeared to be mediated by a negative response element found in the proximal region of the human UCP1 promoter [75]. It is apparent from this study that the vitamin D/VDR complex suppresses the browning process, at least in this specific tissue.

The ability to convert white adipocytes or to manipulate stem cells to result in hyperplasia of brown adipocytes is crucial to the concept of stem cell treatment of obesity and

metabolic syndrome. Transplantamtion of BAT has shown to markedly improve glucose metabolism and insulin resistance in mice [15, 76]. If these findings can be repeated in humans, there is opportunity to ameliorate obesity related disease with therapeutic BAT tissue.

2.2.3 Brown Adipose Tissue Activation

BAT is richly innervated by sympathetic nervous system efferent fibers. The release of noradrenaline from these fibers enhance both the thermogenic activity, via UCP1 activity, and heat production, which involves BAT cell proliferation, mitochondriogenesis, and UCP1 expression [77]. The role of β3 adrenergic receptors has been particularly well characterized in BAT. These receptors are found on the cell surface of mature brown fat cells and are stimulated by norepinephrine as part of a sympathetic nervous system reaction. The stimulation results in the induction of an intracellular pathway activating cyclic adenosine monophosphate (cAMP), protein kinase A, hormone sensitive lipase, lipid oxidation, and downstream kinases and transcriptional factors that mediate UCP1 thermogenic activity [16, 78, 79]. Activation of lipolysis in this process results in degradation of fatty acids mobilized from intracellular triacylglycerol stores and the systemic circulation conferring the lipid regulatory effect of BAT [14, 80]. The Rothwell and Stock calculation estimated that an average human male expending approximately 2500 kcal/day could yield an increase in energy expenditure (EE) by 10-20% provided proper stimulation of 40-50 g of BAT. Some researchers support the Rothwell and Stock calculation by reporting EE increases of 11-34% but other researchers hypothesize and/or report a much more conservative number of 2-5% of total EE [70].

It is well understood that brown adipose tissue is activated by cold exposure. A number of studies report enhanced BAT activity with as little as two hours of cold exposure in both

human and mouse models. One theory suggests that cold stimuli produces vascular endothelial growth factor expression of which yields angiogenesis. This angiogenesis in BAT is associated with BAT hyperplasia [58]. Another theory suggests that transient receptor potential channels (TRP) are responsible for receiving the cold stimuli and inducing thermogenesis via the aforementioned β3 adrenergic mechanism. It has been established that these TRPs receive stimuli from chemical compounds as well.

2.2.4 Regulation of Brown Adipose Mass by Nutritional and Non-nutritional Factors

A variety of nutritional and non-nutritional regulators have been identified in the development and activation of brown adipose mass. These factors encourage the activation of brown adipose tissue, promote the browning of white adipose tissue, or work to achieve both activation and development.

For example, BAT can be activated by various capsinoid analogues like capsaicin found in chili peppers. These compounds increase thermogenesis via the TRPs and β3 adrenergic mechanisms [58]. Moreover, ephedrine [81], caffeine[82], catechin polyphenols[83], and medium-chain triglycerides [84] have also demonstrated β3 adrenergic stimulating or UCP1 stimulating activity [81-85]. Utilization of these dietary compounds has been effective in increasing energy expenditure ranging from 4 to 8% of the total 24-hour energy expenditure [55]. However, is important to note that these particular compounds have alternative properties that may contribute to body composition changes aside from UCP1 stimulation like appetite suppression, fat absorption inhibition, and promotion of physical activity [55].

Other dietary factors known to activate brown adipose tissue include dietary methionine and leucine restriction, fucoxanthin, conjugated linoleic acid, and omega 3 fatty acids [86]. Some

of these aforementioned dietary factors are also capable of stimulating the browning of white adipose tissue.

Both methionine and leucine restriction diets result in the activation of brown adipose tissue. Mice deprived of these essential amino acids experience increased UCP1 induction and β3-adrenergic signaling. The data strongly supports the relationship between methionine and leucine deprivation and weight regulation. It is hypothesized that methionine deprivation increases energy expenditure by accelerating β-adrenergic receptor signaling. Leucine deprivation is thought to reduce fat deposition and support fat loss via activation of BAT and the subsequent catabolism of fatty acids [87-89]. Conversely, there is evidence to suggest that leucine supplementation, rather than deprivation, is beneficial *in vivo*. Supplementation with leucine increased mitochondrial mass and oxygen consumption rates which yielded weight loss or diet-induced obesity resistance [90].

Fucoxanthin, a carotenoid found in brown algae and seaweed extract, has been identified as a nutritional agent of increasing UCP1 and lipid catabolism proteins at both the mRNA and protein expression levels in white adipocytes but not brown adipocytes. Moreover, fucoxanthin metabolites appear to enhance WAT β3-adrenergic receptor production, increasing sensitivity to sympathetic nerve signaling. There is also evidence to suggest that activation of AMPK, fatty acid β-oxidation, and lipogenesis results from the presence of fucoxanthin. The specific mechanisms for the metabolite effects on WAT browning have yet to be elucidated [91-95].

Like fucoxanthin, 2-hydroxyoleic acid, a synthetic olive oil analog, was able to induce UCP1 expression in white adipose tissue selectively. Likewise, this analog has been shown to enhance the cAMP/PKA pathway, resulting in the browning of white adipose tissue. Oral

supplementation with natural olive oil reportedly increases expression of all uncoupling proteins and promotes escalated total body oxygen consumption [96-100].

The trans-10, cis-12 isomer of conjugated linoleic acid (CLA) has shown marked adipose reduction in variety of species by increasing WAT apoptosis, decreasing differentiation and lipogenesis of adipocytes, and increasing fatty acid oxidation. Research has also described the ability of CLA to increase brown phenotypic genes including UCP1, PGC1α, and Cidea in WAT specifically [101-103].

Lastly, omega 3 fatty acids have shown the capacity to mediate adipose function and growth by both up-regulation of UCP1 and WAT oxidation [104, 105]. Alternately, another study found that just marginal amounts of EPA and DHA in the daily diet, comprising just 15% of daily dietary fats, can increase β-oxidation in conjunction with the up-regulation of other PGC1α, CPT1, and Nrf1 but not UCP1 genes in WAT [106].

Various non-nutritional regulators are also key in promoting the development and activation of brown adipose tissue [58]. For example, exercise acts as a non-nutritional regulator resulting in the browning of white adipose tissue. It is said to increase the expression of Irisin, a PGC1α dependent myokine that induces the browning of white adipose tissue [58]. This phenomenon may contribute to the overall effect of regular exercise on increasing basal metabolic rate.

Other identified non-nutritional regulators of browning include: cyclooxygenase-2, receptor interacting protein 140, liver X receptor α, bone morphogenetic protein 7, fibroblast growth factor 21, and retinoblastoma protein among others [86].

2.3 The Research Gap

In summary, the existing research exploring the role of vitamin D_3 in total energy metabolism is contradictory and inconclusive. *In vitro* white adipocyte study results would suggest that vitamin D_3 impairs growth by inhibiting the production of its major regulators PPARγ and C/EBPα. Conversely, the *in vivo* data would suggest that vitamin D_3 promotes WAT differentiation and growth because VDR ablation decreased body fat [26] while VDR over expression increased body fat [52].

The effect of vitamin D_3 on adipose differentiation and function has only been studied in WAT. The purpose of this thesis is to characterize the effect of both over expression of the VDR and an over abundance of vitamin D_3 in brown adipose differentiation and mitochondrial respiration. A characterization of the effects of vitamin D_3 on brown adipocytes will provide a better understanding of the environment in which BAT grows and functions optimally. To measure the effect of vitamin D_3 on differentiation the following methods were used: mRNA expression was to quantify specific gene expression, Oil Red O stain to determine the change in lipid accumulation, MitoTracker to quantify mitochondrial content, and western blot to detect proteins. To measure the effect of vitamin D_3 on mitochondrial respiration, a mitochondrial stress test was conducted and analyzed. Lastly, a luciferase assay was used to determine changes in PPARγ transactivation under the influence of both the vitamin D_3 ligand and overexpression of VDR.

CHAPTER III
Materials and Methods

3.1.1 Reagents

Vitamin D_3, $1,25(OH)_2D_3$, was purchased from Enzo (Farmingdale, NY). 3-isobutyl-L-methylxanthine, T_3, dexamethasone, insulin, and indometacin were purchased from Sigma-Aldrich (St. Louis, MO).

3.1.2 Animals

The mice studies were approved by the Institutional Animal Care and Use Committee at the University of Tennessee. Diet-induced obesity (DIO) mice, genetically obese ob/ob (leptin deficient) mice, and their respective controls have been described elsewhere[107]. Briefly, the mice were purchased from the Jackson Laboratory (Bar Harbor, ME). For DIO study, 6-week old male C57BL/6J mice (n=7 per group) were fed with either a high-fat diet (60% Kcal from fat, Research Diets Inc., New Brunswick, NJ) (DIO group) or chow diet (Chow group) for 20 weeks before sacrificed at 26 weeks of age. For ob/ob mice study, 6-week old male ob/ob (Ob/ob group) and their control wild type mice (Wt group) (n=6 per group) were fed with chow diet for 8 weeks before sacrificed at 14 weeks of age. Upon sacrifice, interscapular BAT was removed and immediately snap frozen in liquid nitrogen and stored at -80 °C until analysis.

3.1.3 Brown fat cell culture and differentiation

Murine brown fat cell line is a gift from Dr. Johannes Klein (University of Lubeck, Lubeck, Germany), who has generated the cell line from interscapular brown fat of newborn

C57BL/6 mice[108]. Brown fat cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS, Atlas Biologicals, Fort Collins, CO) at 37°C humidified atmosphere of 5% CO_2 in air, until they reached confluence (designated as day 0 or D0). The cells were then induced to differentiate by the induction media containing DMEM supplemented with 20% FBS, 1 nM T_3, 20 nM insulin, 0.125 mM indometacin, 5 μM dexamethasone, and 0.5 mM 3-isobutyl-L-methylxanthine for 24 hr, followed by changing into the differentiation media containing DMEM supplemented with 20% FBS, 1 nM T_3, and 20 nM insulin every two days until fully differentiated on day 6 (D6). For the study of $1,25(OH)_2D_3$ on brown adipocyte differentiation, $1,25(OH)_2D_3$ (1, 10, 100 nM) or the vehicle control DMSO was added at D0, and replaced with each change of the media during the differentiation process, or otherwise as indicated in the figure legends. For the study of VDR over expression, Myc-DDK tagged murine VDR expression plasmid (OriGene, Rockville, MD) was transfected into brown preadipocytes for 72 hr and the stably transfected cells were selected by antibiotics (G418) for 3 weeks. The exogenous VDR expression was confirmed by mRNA and protein expression.

3.1.4 Western Blot Analysis

Total cell lysates were prepared and protein concentrations were determined by BCA assay kit (Thermo Scientific, Waltham, MA). Thirty micrograms of total cell lysate was subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 20 mM Tris-HCl, 137 mM NaCl, and 0.1% Tween 20 (pH 7.4) containing 5% nonfat milk. The membrane was immunoblotted with primary antibodies at 4 °C for overnight followed by secondary antibody conjugated with horseradish

peroxidase (GE Healthcare, Pittsburgh, PA). The signal was quantified by densitometry using a ChemiDocXRS+ imaging system with ImageLab software (Bio-Rad).

3.1.5 RNA preparation and quantitative real time PCR analysis

Total RNA was prepared using TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Total RNA abundance was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Reverse transcription was carried out using Fermentas first strand synthesis kit (Thermo Scientific, Pittsburgh, PA) according to the manufacturer's instructions. mRNA expression of indicated genes and the house keeping 36B4 were measured quantitatively using Absolute Blue QPCR SYBR Green ROX mix (Thermo Fisher Scientific) and gene specific primers (primer sequences available upon request). PCR reactions were run in a 96-well format using an ABI 7300HT instrument. Cycle conditions were 50 ^{0}C for 2 min, 95 ^{0}C for 15 min, then 40 cycles at 95 ^{0}C for 15 s/60 ^{0}C for 1 min. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, which normalizes against 36B4.

3.1.6 MTT Assays

The assays for measuring cell viability were performed according to the manufacturer's instructions. Briefly, the brown preadipocytes were seeded into 24-well plates. When reached confluency, they were induced to differentiate according to the procedures described above in the presence or absence of Vit D_3 (1, 10, 100 nM) for 24 hr, 48 hr, and 5 days. At the end of the indicated times, 50 μL of MTT (5 mg/mL) was added to each well and the plate was incubated at

37 °C for 3 hr. Purple formazan crystal was solubilized by addition of 100 μL isopropanol. Absorbance was measured at a test wavelength of 570 nm and a reference wavelength of 650 nm using GloMax-Multi Detection System.

3.1.7 Analysis of mitochondrial content by MitoTracker green staining

Mitochondrial contents were labeled using mitochondria-specific dye MitoTracker Green (Life technologies, Carlsbad, CA) according to manufacturer's protocol. Briefly, the differentiated brown adipocytes were washed and trypsinized from the cultured plate and incubated with mitoTraker green at 100 nM for 30 min at 37 ^{0}C. The fluorescence intensity was measured with Accuri C6 flow cytometry (BD, Franklin Lakes, NJ). Background autofluorescence from non-stained cells were averaged and subtracted from the mean fluorescence intensity values. Relative fluorescence intensity is the fold of the mean fluorescence intensity of the controls.

3.1.8 Reporter Gene Assays

Brown preadipocytes were transiently transfected with PPARγ transactivation reporter system [109], in which murine PPARγ ligand binding domain is coupled to the Gal4 DNA binding domain to form mPPARγ-Gal4 and a reporter construct containing an upstream activating sequence (UAS)–linked luciferase, 4xUAS-TK-luc (TK: thymidine kinase) and β-galactosidase expression plasmid. PPARγ transactivation system was a gift from Dr Susanne Mandrup at University of Southern Denmark, Denmark. The cells were then pre-treated with increasing doses of $1,25(OH)_2D_3$ (1, 10, 100 nM) or the vehicle control DMSO overnight and

then co-treated with or without PPARγ ligand rosiglitazone (1 μM) for 8 hr. In the VDR over expression study, PPARγ transactivation system and β-galactosidase expression plasmid were co-transfected with murine VDR expression plasmid or the vector plasmid for 24 hr. The cells were then treated with or without PPARγ ligand rosiglitazone for 8 hr. The cell lysate was prepared and reporter luciferase and β-galactosidase activities were measured with GloMax Luminometer (Promega, Madison, WI). Relative luciferase activities were normalized by β-galactosidase activities and expressed as fold of the vehicle control.

3.1.9 Cell respiration measurements

$5x10^4$ brown adipocytes (D6) that have been treated with or without 1, $25(OH)_2D_3$ (1, 10, 100 nM) were sub-cultured in 24 well XF assay plates overnight in the differentiation medium and subjected to real-time measurements of oxygen consumption (OCR) and extracellular acidification of the medium (ECAR) using XF24 Extracellular Flux Analyzer (Seahorse Biosciences, North Billerica, MA). Cells were rinsed once, changed to 500 μl of XF assay buffer (DMEM without $NaHCO_3$, containing 10 mM glucose, 2 mM pyruvate, 2 mM GlutaMAX, pH 7.4), and equilibrated at non-CO_2 incubator and 37 ^{0}C for 1hr. Following the basal measurements of OCR and ECAR, all cells were injected with isoproterenol (1 μM) and subsequent readings were taken over a 6 hr-period. For mitochondrial stress tests, mitochondrial complex inhibitors were injected to all treatments sequentially in the following order: oligomycin (1 μM), FCCP (0.75 μM), antimycin A/rotenone (1 μM each), and 3 readings were taken after each inhibitor. OCR and ECAR were automatically recorded by XF24 software v1.8 provided by the

manufacturer. Calculations of maximal OCR, OCR from ATP generation, and from proton leak, and spare respiration capacity were performed according to the manufacturer's instructions.

3.1.10 Statistical Analysis

Statistical analysis was performed using SigmaPlot 11.0 (Systat Software, Inc.). One way ANOVA with repeated measures followed by multiple comparisons test (Student-Newman-Keuls Method) were performed to determine the differences among the treatment groups (e.g., doses) and/or time points (e.g., D0-D6). Student's t-tests were performed when appropriate. Data were Log transformed when appropriate. The level of significance was set at $p < 0.05$.

CHAPTER IV
Results and Discussion

4.1 Results

4.1.1 VDR, CYP24A1 and CYP27B1 mRNA expression in the BAT of mice model of obesity and during brown adipocyte differentiation *in vitro*

First, mRNA expression of VDR, vitamin D_3 activating enzyme 1α-hydroxylase CYP27B1, and degrading enzyme 24-hydroxylase CYP24A1 in the interscapular BAT of both diet-induced obesity and genetically obese ob/ob (leptin deficient) mice was examined. Expression of UCP1 mRNA was significantly lower in the BAT of ob/ob mice when compared to the wild type counterpart ($p<0.05$) (Fig. 2A). The mRNA of both VDR and CYP27B1 in the BAT of ob/ob were not statistically significant. In the DIO mice model of obesity, no significant changes were seen in mRNA levels of VDR, CYP27B1, and UCP1. Levels of CYP24B1 mRNA were undetectable by q-PCR (Fig. 2B).

4.1.2 Effects of $1,25(OH)_2D_3$ on the vitamin D system during brown adipocyte differentiation

To study the vitamin D_3 system in brown adipocyte differentiation, an immortalized brown fat cell line generated from the classical interscapular BAT from C57BL/6 mice [108] was utilized and mRNA expression of VDR, CYP27B1, and CYP24A1 during the differentiation process was examined. VDR mRNA expression was induced by the induction treatment at day 1 (D1), and gradually decreased to less than 10% of the D0 level (Fig. 3A). A similar mRNA profile of induction at D1 and subsequent decrease was seen in CYP24A1 (Fig. 3B). The induction treatment did not result in induction of CYP27B1; however, its expression was

diminished from the basal rate by the end of differentiation (Fig. 3C). The results suggest that vitamin D_3 system is down-regulated in brown adipocyte differentiation.

4.1.3 Effects of $1,25(OH)_2D_3$ on brown adipocyte lipid accumulation, mRNA expression, and mitochondrial content

Brown preadipocytes were differentiated under the vehicle control DMSO or under conditions described in materials and methods with variable doses of $1,25(OH)_2D_3$ (1, 10, or 100 nM) for seven days (D0-D6) (Fig. 4A top). Cell viability (MTT) assays indicated that $1,25(OH)_2D_3$ did not affect cell viability when treated up to 100 nM for up to 5 days (data not shown). The ORO stains reflect dose-depended suppression of brown adipocyte lipid accumulation by $1,25(OH)_2D_3$ (Fig. 4A-C).

Moreover, the data shows a dose dependent suppression in mRNA expression of brown adipocyte markers peroxisome proliferator activator receptor gamma (PPARγ), peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α), uncoupling protein 1 (UCP1), nuclear respiratory factor 1 (Nrf-1), cell-death inducing DFFA-like effector a (Cidea), and nucleus encoded mitochondrial gene, cytochrome c oxidase subunit IV a (Cox4a) (Fig. 4D). $1,25(OH)_2D_3$ degrading enzyme CYP24A1 mRNA was dose-dependently up-regulated, validating the presence of $1,25(OH)_2D_3$ in the treatment due to the understanding that increased $1,25(OH)_2D_3$ yields an increase in its degrading enzyme to dispose of it (Fig. 4D). It should be noted that during the course of the differentiation, the most suppressive effects of calcitriol were seen at D4 and either diminished slightly or plateaued at D6. Thus, D4 will be the time point used in the subsequent VDR experiments. Protein expression of PPARγ, PGC-1α, and UCP1

confirmed 1,25$(OH)_2D_3$'s dose-dependent effects on mRNA expression during differentiation (Fig. 4E).

With the understanding that 1,25$(OH)_2D_3$ treatment inhibits mRNA expression of PGC-1α, the master regulator of mitochondrial biogenesis, and other mitochondrial genes, such as Nrf-1, Cox4a we chose to further explore the impact on mitochondrial biogenesis. To confirm or deny that reduction in gene expression resulted in impaired mitochondrial biogenesis, we employed mitochondrial specific fluorescence dye mitoTracker green to measure mitochondrial content, i.e. number of mitochondria. Consistent with the mRNA results, 1,25$(OH)_2D_3$ significantly suppressed mitochondrial content on D4, but not on D6 (Fig. 4F).

4.1.4 Brown adipocyte time sensitivity to 1,25$(OH)_2D_3$ treatment

To better understand time sensitivity of 1,25$(OH)_2D_3$ treatment, we differentiated brown preadipocytes in the presence of 1,25$(OH)_2D_3$, either starting from day 0 (Day 0-6), day 3 (Day 3-6) or from day 5 (Day 5-6). The full 7 day treatment resulted in the most significant suppression on differentiation, compared to those of Day 3-6 and Day 5-6 treatment (Fig. 5A). The next time sensitivity experiment measured differentiation of brown preadipocytes with1,25$(OH)_2D_3$ for an allotted time frame, being introduced and removed at different stages of differentiation. When 1,25$(OH)_2D_3$ was not present, cells were provided standard media. The Day 0-1 and Day 0-2 treatments resulted in a similar level of suppression to that of the full Day 0-6 treatment from the previous experiment. The Day 3-4 or Day 5-6 treatments were ineffective in attenuating differentiation (Fig. 5B). These data suggest that first days of differentiation (Day 0 to Day 2) are the most sensitive to 1,25$(OH)_2D_3$ treatment.

4.1.5 Effects of $1,25(OH)_2D_3$ on mitochondrial respiration in brown adipocytes

To confirm the functional consequences of the effect of vitamin D_3 on brown adipocyte differentiation, we examined mitochondrial respiration in $1,25(OH)_2D_3$ treated brown adipocytes by measuring cellular bioenergetics coupled with mitochondrial stress tests using a XF24 Extracellular Flux Analyzer (Fig. 6A-B). Isoproterenol-stimulated oxygen consumption rates (OCR) (Fig. 6C), maximal OCR (Fig. 6D), and OCR from proton leak (Fig. 6F) were significantly impaired by $1,25(OH)_2D_3$ in concentrations as low as 1 nM ($p<0.01$). However, $1,25(OH)_2D_3$ treatment did not impact ATP-generating OCR (Fig. 6E) and spare respiration capacity (Fig. 6G) even at the highest concentration.

4.1.6 Effects of over-expression of VDR on brown adipocyte differentiation

Since our preliminary experiments showed down-regulation of VDR during differentiation we chose to artificially increase VDR, employing a stable transfection of murine VDR expression plasmid into the brown preadipocytes. Measurements were taken at day 4 of differentiation because this time point seemed to be the most influenced by the vitamin D system as evidenced by the previous mRNA study (Fig 4). Pooled VDR transfected brown preadipocytes, in the absence of $1,25(OH)_2D_3$, showed a two-fold increase in mRNA expression of VDR, which correlated to a ~ten-fold mRNA expression of CYP24A1 when compared vector-transfected pool cells (Fig. 7A). Conversely, VDR over-expression yielded no change of CYP27B1 mRNA compared with the control (Fig. 7A). VDR over-expression suppressed mRNA expression of the brown adipocyte markers UCP1 (Fig. 7B), PGC-1α (Fig. 7C), and PPARγ (Fig. 7D) at basal (D0) and D4 in the differentiation process. These results are consistent with the effects of $1,25(OH)_2D_3$ treatment in the aforementioned experiments.

4.1.7 1,25(OH)$_2$D$_3$/VDR suppresses PPARγ transactivation in brown preadipocytes

As described previously, PPARγ transactivation is necessary for successful differentiation of brown preadipocytes into mature, functional adipocytes. To fully understand the mechanism by which 1,25(OH)$_2$D$_3$/VDR suppresses differentiation, we measured PPARγ transactivation via reporter assays. Treatments of 1, 10, and 100 nm of 1,25(OH)$_2$D$_3$ dose-dependently suppressed both basal and PPARγ ligand rosiglitazone (Rosi, 1μM)-induced PPARγ transactivation (both $p<0.05$ for the trend) (Fig. 8A). Consistently, Rosi-induced PPARγ transactivation in VDR transgenic mice was similarly suppressed ($p<0.05$) (Fig. 8B).

4.2 Discussion

Accumulating evidence suggests the critical involvement of the vitamin D$_3$ system in energy metabolism. The majority of existing studies evaluate the effects of vitamin D$_3$ on adipogenesis and lipid metabolism in white adipose tissue, and/or white adipocytes [110]. In the *ob/ob* mouse model, UCP1 was significantly decreased (Fig. 2A). However, neither VDR and CYP27B1 were altered (Fig. 2A). Similarly, in the diet induced obesity mouse model, mRNA of UCP1, CYP27B1, CYP24A1 expression were not significantly different from that of the chow-fed mice (Fig. 2B). The current research indicates mixed effects of diet-induced obesity on UCP1 expression. Excessive caloric intake resulting in obesity has been associated with increases, decreases, and no effect on UCP-1 expression [111]. The impaired UCP1 mRNA expression in the BAT of ob/ob mice of the current study lead to inquisition about the potential correlation between obesity and an impaired energy metabolism due to reduced BAT efficiency. The

vitamin D/VDR complex was explored for its potential to either ameliorate or exacerbate BAT differentiation and function because of its previously identified relationship with WAT.

Potential mechanisms underlying altered energy metabolism in VDR knockout mice [26] and transgenic mice with adipose-specific over-expression of VDR have been elucidated [52]. To recap, such mechanisms include altered UCP1 expression and modified metabolic markers such as adiponectin and insulin. The results demonstrate the effects of $1,25(OH)_2D_3$ to dose-dependently suppress brown adipocyte differentiation, as evidenced by impaired lipid accumulation and brown marker gene expression, including the expression of PPARγ, PGC-1α, and UCP1. The mechanisms of such suppression have been explored. As described previously, both white and brown adipocyte differentiation rely on PPARγ for proper differentiation [112-114]. Moreover, PPARγ transcriptional co-activator PGC-1α is crucial in brown adipocyte differentiation and mitochondrial gene control in response to cold exposure [115]. As early as day 2 (D2) in the differentiation process, $1,25(OH)_2D_3$ dose-dependently suppressed both mRNA and protein expression of PPARγ and PGC-1α (Fig. 4D-E). These data indicate that down regulation of these key factors elicit suppressive effects on brown adipocyte maturity. Consistently, VDR over expression also inhibited basal (D0) mRNA of PPARγ and PGC-1α (Fig. 7B). When using PPARγ transactivation reporter assays, it was found that both $1,25(OH)_2D_3$ and VDR over expression suppressed basal and/or ligand-induced PPARγ transactivation (Fig. 8A-B), consistent with the effects of $1,25(OH)_2D_3$ on 3T3-L1 adipocyte differentiation [28]. It is believed that an abundance of $1,25(OH)_2D_3$ or VDR competes with PPARγ for its dimerization partner retinoid X receptor, thereby suppressing PPARγ

transactivation [28]. Presumably, this competition for RXR is the mechanism responsible for the $1,25(OH)_2D_3$/VDR signal suppression of brown adipocyte differentiation.

When comparing the suppressive effects of $1,25(OH)_2D_3$ on brown adipocyte marker gene expression and mitochondrial contents (Fig. 4D-F) during the 6 day span of differentiation, there is a notable trend of maximum suppression at D4 and recovery on D6. PGC-1α expression was maximally suppressed at D4 but the suppression was diminished by D6 (Fig. 4D-E). This expression pattern was consistent with the changes of mitochondrial content by $1,25(OH)_2D_3$ (Fig. 4F). As mature adipocytes at D6, only protein expression of PPARγ, but not PGC-1α, was suppressed. Suppressed PPARγ may explain the suppression of UCP1 since PPARγ is required for normal BAT differentiation which includes the expression of its hallmark protein, UCP1. Cellular bioenergetics results revealed that maximal OCR and OCR from proton leak (i.e., uncoupled respiration) (Fig. 6D, F), but not ATP-generating OCR and spare respiration capacity (Fig. 6E, G), were affected by $1,25(OH)_2D_3$. These data are consistent with $1,25(OH)_2D_3$-diminished UCP1 expression, not of mitochondrial content, at D6. The gene expression results of $1,25(OH)_2D_3$ on UCP1 expression agree with previous findings of increased UCP1 mRNA in the BAT of VDR knockout mice and decreased UCP1 mRNA in the BAT of transgenic mice of adipose-specific VDR over-expression. Despite the published results and the current findings, the impact of $1,25(OH)_2D_3$/VDR on mitochondrial content and PGC-1α expression in the BAT of these mice models is unclear. Whether $1,25(OH)_2D_3$/VDR modulates UCP1 expression and brown adipose tissue development through direct effects or through modulation of mitochondrial biogenesis (via PGC-1α) in brown adipose tissue *in vivo* remain to be determined. In addition,

differential effects of $1,25(OH)_2D_3$ on PPARγ as opposed to PGC-1α also need to be measured in the future.

Apart from classical brown adipocytes present in BAT, "browning" of WAT indicated by adoption of a brown phenotype (i.e. UCP1 expression) in response to specific stimuli such as chronic cold exposure and β-adrenergic stimulation, has been reported in animal models. Due to the potential for energy modulation, mechanisms underlying the browning process have been a the target of investigation [116]. Thus, discovering that UCP1 mRNA and protein were significantly elevated in WAT of VDR knockout mice [117] suggests that VDR may be a negative regulator for WAT browning. It would be beneficial to examine the impact of the $1,25(OH)_2D_3$/VDR system in cultured BAT progenitor cells and in animal models.

The existing data describing the suppressive effects of the vitamin D/VDR complex in 3T3-L1 cell differentiation [27, 28] in combination with the current findings revealing similar suppression in brown adipocytes seems to contradict the results of *in vivo* studies. In my studies, the vitamin D/VDR complex impaired both development and UCP1 expression. When compared to wild type mice, VDR knockout mice presented with decreased white and brown adipose masses but presented with increased UCP1 in the BAT [30, 117]. Alternatively, VDR over-expression in transgenic mice [31] had increased brown and white adipose tissue masses but recorded decreased UCP1. Whether or not the exchange of enlarged BAT mass for enhanced UCP1 expression, and vice versa, yields the same power of thermogenesis and energy expenditure is unknown. Moreover, the reasons and molecular basis for the inconsistency between *in vitro* and *in vivo* results are unclear. It appears that both the global inactivation of VDR and global over expression of VDR starting from embryonic stages of BAT development does not in produce the exact opposite of $1,25(OH)_2D_3$/VDR on brown adipocyte differentiation

in cultured cells. The generation and characterization of mice with brown adipose-specific and/or temporally inducible VDR knockout mice will be informative in understanding the role of $1,25(OH)_2D_3$/VDR on BAT development and function *in vivo*.

Our research provides advanced understanding of the suppressive effects of the $1,25(OH)_2D_3$ /VDR complex on the differentiation brown adipocytes and the functionality of mice-derived brown adipocyte cell line. Moreover, our results suggest critical role of both the vitamin D_3 ligand and VDR in modulation of BAT development and function in models of obesity.

4.3 Future Directions

Both clinical and epidemiological human studies correlate serum $25(OH)D_3$ with obesity as determined by BMI, fat mass, and waist circumference [110]. Low serum $1,25(OH)_2D_3$ levels are associated with higher BMI in healthy and obese individuals [118]. However, because of the fat solubility of vitamin D_2 and vitamin D_3, it is unclear whether the low serum levels of $25(OH)D_3$ and $1,25(OH)_2D_3$ reflect the total levels of vitamin D_3, or are unfair representations due sequestration of various forms of vitamin D_3 in the fat tissue. For this reason, further investigation must be initiated to understand the true availability of vitamin D and its analogs. With true understanding of vitamin D availability in adipose tissue, one can better understand the relationship between vitamin D status and the development and function of WAT and BAT.

Moreover, a review of the literature strongly suggests that moderate doses (i.e., 10^{-8} mM or 10 nM) of $1,25(OH)_2D_3$ suppresses 3T3-L1 adipocyte differentiation [27, 28]. However, it has been reported that a similar doses of $1,25(OH)_2D_3$ actually *promote* the differentiation of human subcutaneous preadipocytes [29]. Clearly, more research is required to definitively describe the

role of $1,25(OH)_2D_3$/VDR in human brown adipocyte differentiation and in the modulation of both WAT and BAT in the development of obesity in humans.

In light of my results, the next logical step would be to test my hypothesis and confirm my findings in an *in vivo* mouse model. It will be informative to determine if a diet enriched with vitamin D would modulate the mass and function of BAT as well as WAT. The experimental mice will be fed with the current daily allowance recommendation of 600 IU or 15mcg [119, 120], modified to be appropriate for the body mass of a mouse. The total mass of BAT and WAT will be determined together with whole body energy expenditure and other markers indicative of metabolic health (e.g. fasting glucose level, lipid panels, glucose tolerance and insulin sensitivity among others). It would be useful if these studies were conducted in models of both diet-induced obesity and genetic obesity to examine the *in vivo* role of the vitamin D system in BAT of obese animals.

Another route of exploration would be to knockdown VDR *in vivo*. Ideally, an agent that with BAT specificity would be created to antagonize the vitamin D_3 ligand or to knockdown the expression of VDR in this tissue so to promote the beneficial effects on BAT but avoid the side effects of vitamin D_3 deficiency in other various tissues. Thus, promoting the beneficial effects in BAT while preserving the activity and function in the remaining tissues and organs of the body. Upon knockdown, measurements of the subsequent changes in BAT function and metabolic health of the mouse would be recorded.

With success of this study, the hypothesis would then require a human study. Measurements of adipose tissue (as opposed to serum) vitamin D status, body weight, energy expenditure, brown adipose mass and activation, and browning of WAT after VDR knockdown would be required to draw any conclusions regarding the capacity for vitamin D to manipulate

BAT development and function. If the results yield a positive effect in human metabolic health, targeted therapy options could then be developed.

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APPENDIX

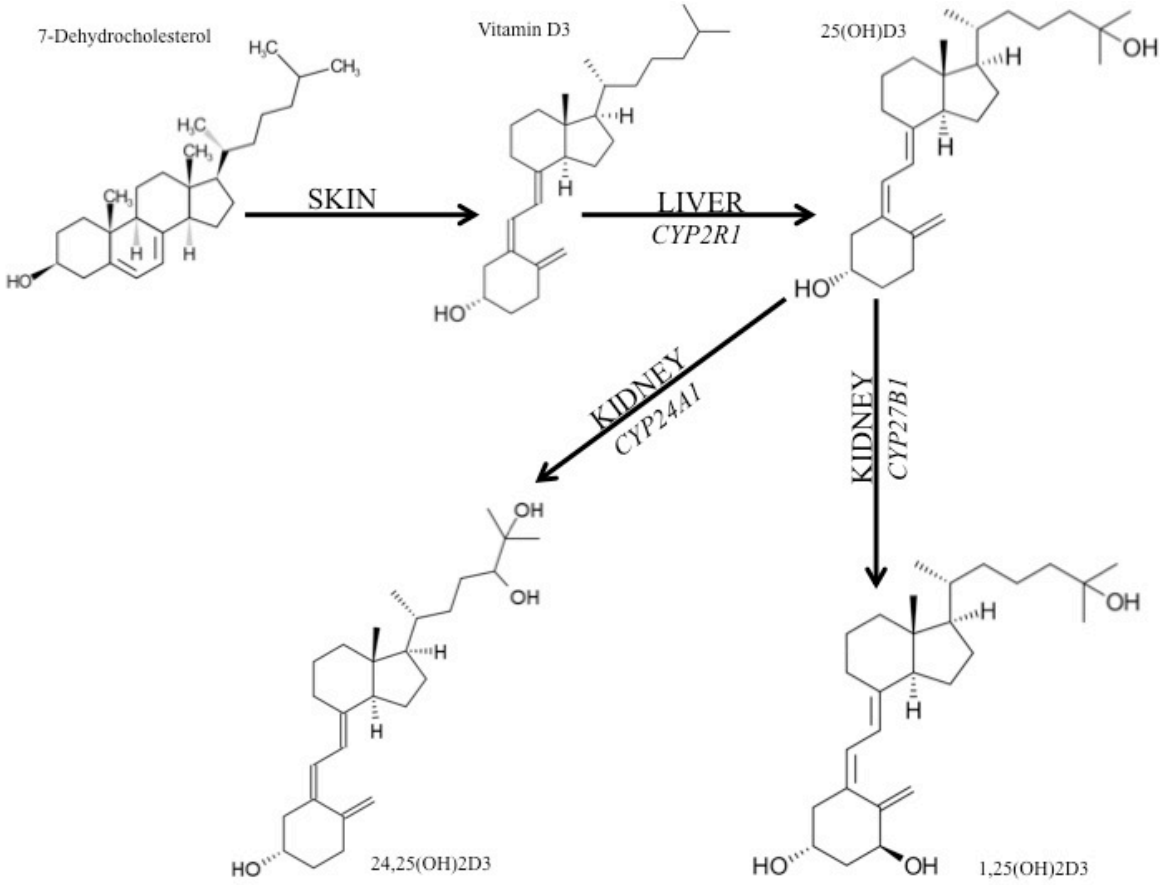


Fig. 1 Endogenous metabolism of Vitamin D_3
When exposed to ultraviolet light, precursor 7-dehydrocholesterol in the skin is structurally changed. The change allows for transportation in the blood to the liver for hydroxylation at the 25th carbon, yielding $25(OH)D_3$, the major circulating form of vitamin D. $25(OH)D_3$ is then hydroxylated at the 1st or 24th carbon, resulting in either $1,25(OH2)D_3$ or $24,25(OH)2D_3$ respectively, determining the fate of the vitamin.

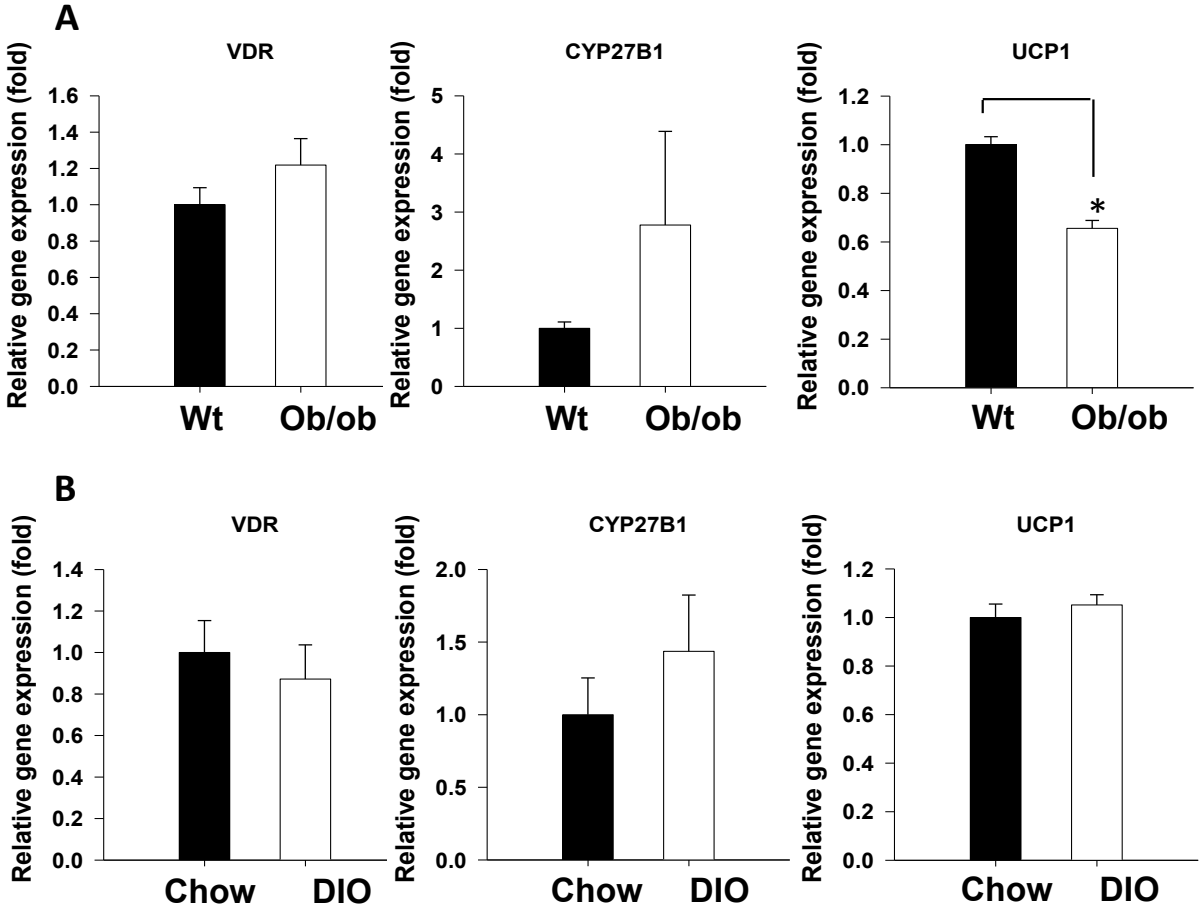


Fig. 2 mRNA expression of VDR and CYP27B1 in mice models of obesity mRNA expression of VDR and CYP27B1 of the BAT from the male ob/ob or wild type control mice (n=6) (A) and from the male DIO or chow-fed control mice (n=7) (B) were analyzed. Relative mRNA expression was normalized to 36B4 and expressed as fold of the controls (set as 1). Data are mean±SE (n=6 or 7). *, significantly different from the controls with $p<0.05$.

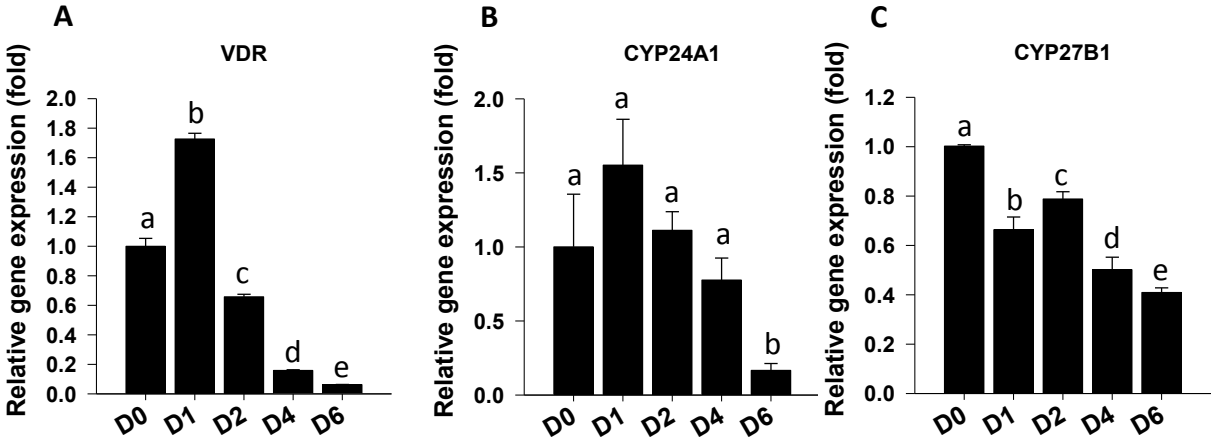


Fig. 3 mRNA expression of VDR, CYP24A1 and CYP27B1 during brown adipocyte differentiation
Brown preadipocytes were differentiated into adipocytes in culture within 6 days. mRNA expression of VDR (A), CYP24A1 (B), and CYP27B1 (C) at day 0 (D0), 2 (D2), 4 (D4) and 6 (D6) were analyzed. Relative mRNA expression was normalized to 36B4 and expressed as fold of D0 value (set as 1). Data are mean±SE (n=3). Different letters indicate significant difference ($p < 0.05$).

Fig. 4 1,25$(OH)_2D_3$ dose-dependently suppresses brown adipocyte differentiation
Brown preadipocytes were differentiated in the presence of increasing doses of 1,25$(OH)_2D_3$ (1, 10, 100 nM) or the vehicle control DMSO as shown in the top panel of (A). Oil red O (ORO) stained lipid accumulation is shown in the low panel of (A). ORO stained cell morphology is shown in (B). ORO absorbance was measured and plotted in (C). Different letters indicate significant difference ($p < 0.05$) in (C). mRNA expression of brown adipocyte markers PPARγ, PGC-1α, PPARγ, UCP-1, Nrf-1, Cidea, Cox4a, and Vit D_3 target gene CYP24A1 were analyzed (D). Relative mRNA expression was normalized to 36B4 and expressed as fold of D0 value (set as 1). (E) Protein expression of PPARγ, PGC-1, and UCP-1 was analyzed by western blot. (F) Mitochondrial content was analyzed by mitochondrial specific fluorescence dye mitoTracker green at D4 and D6. Relative fluorescence intensity was calculated from the mean fluorescence intensity values of the samples and expressed as fold of the controls.

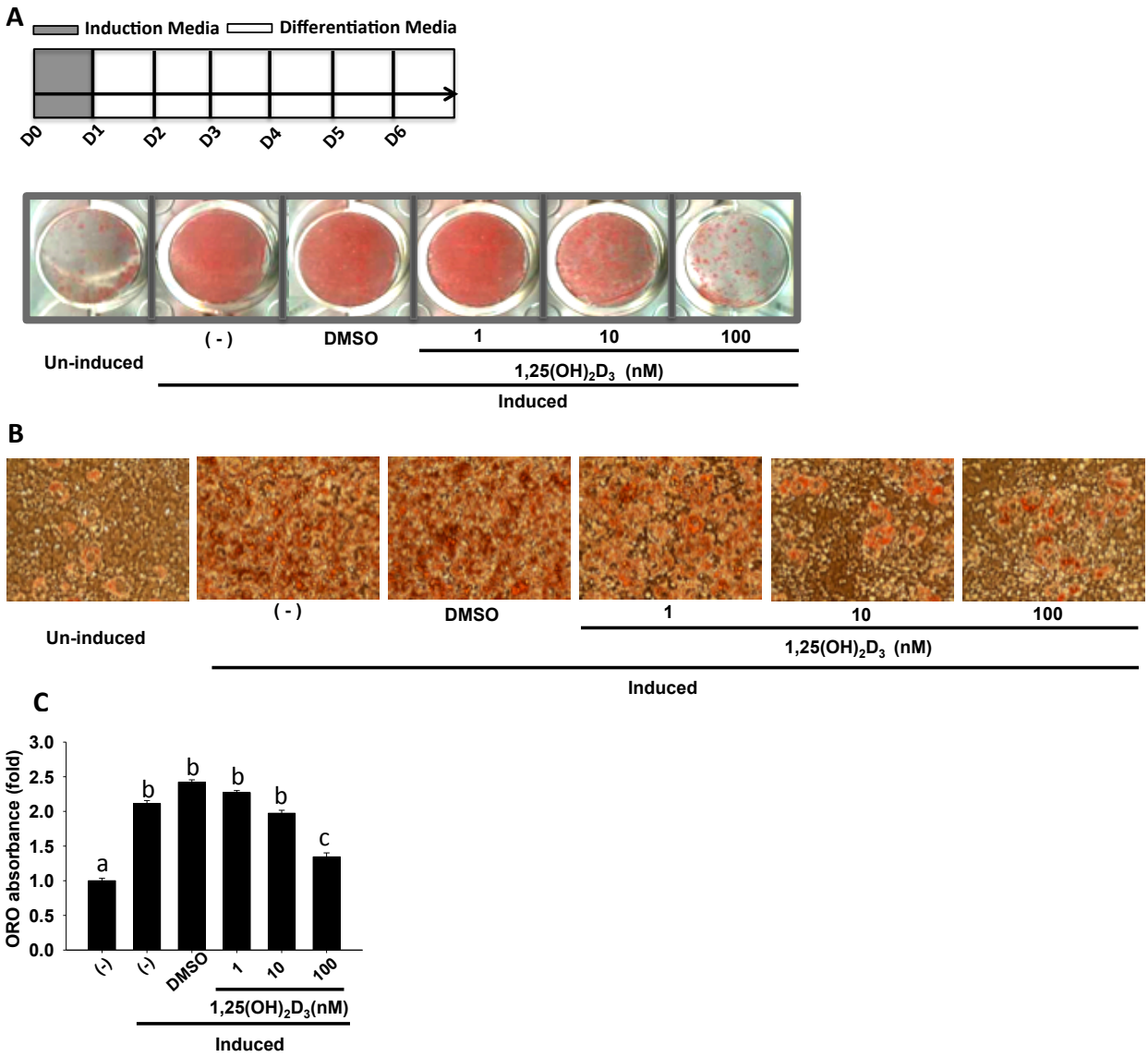
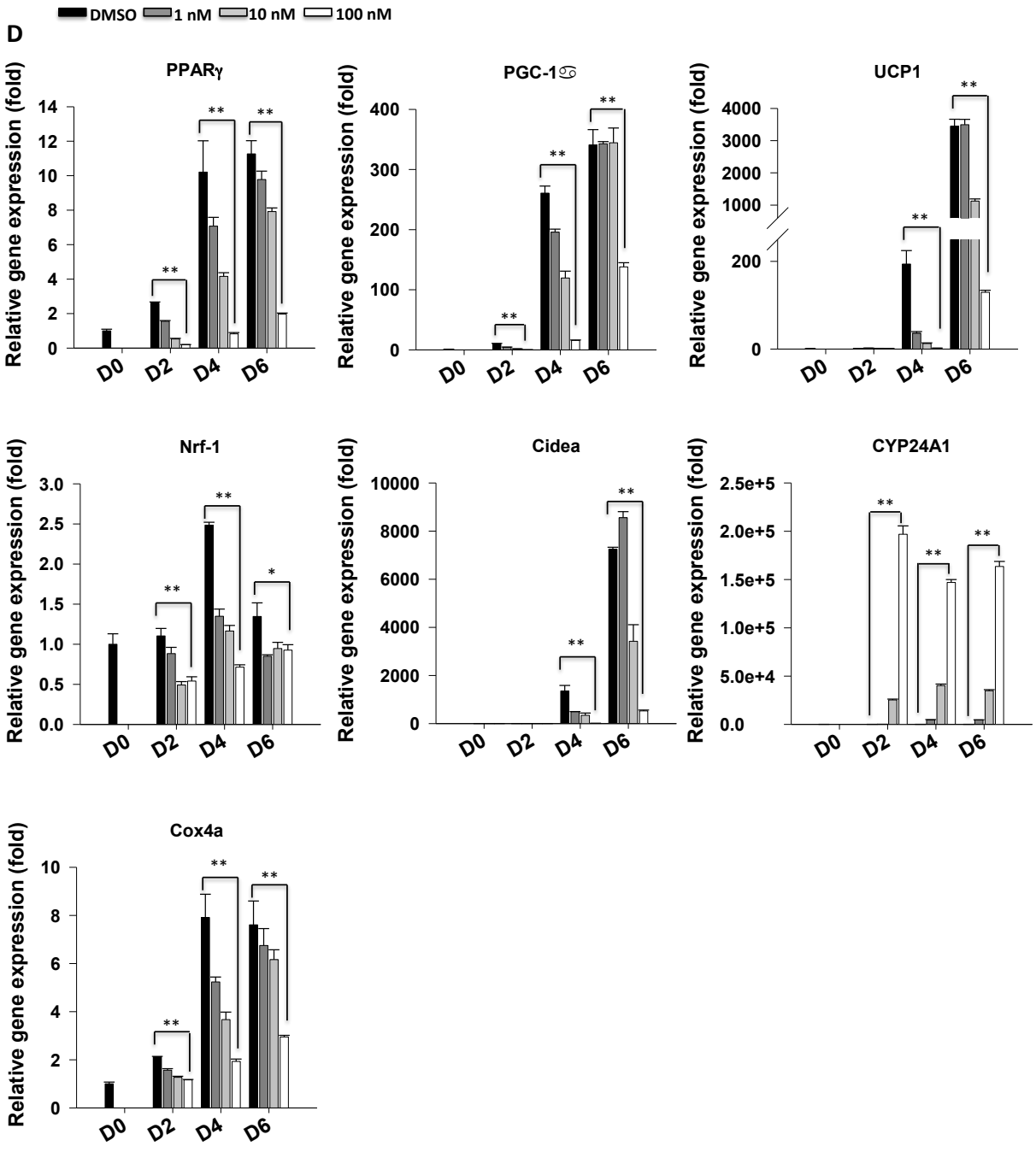


Fig. 4 Continued



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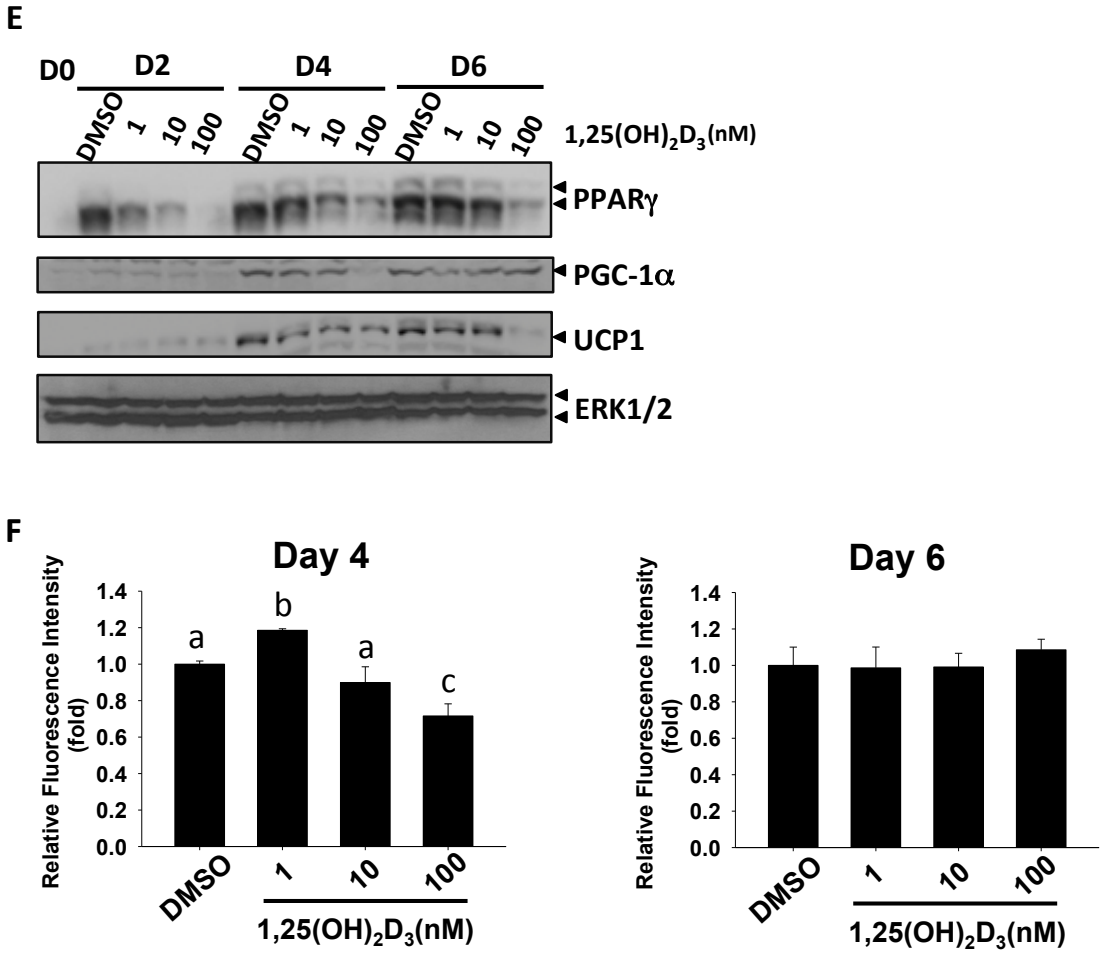

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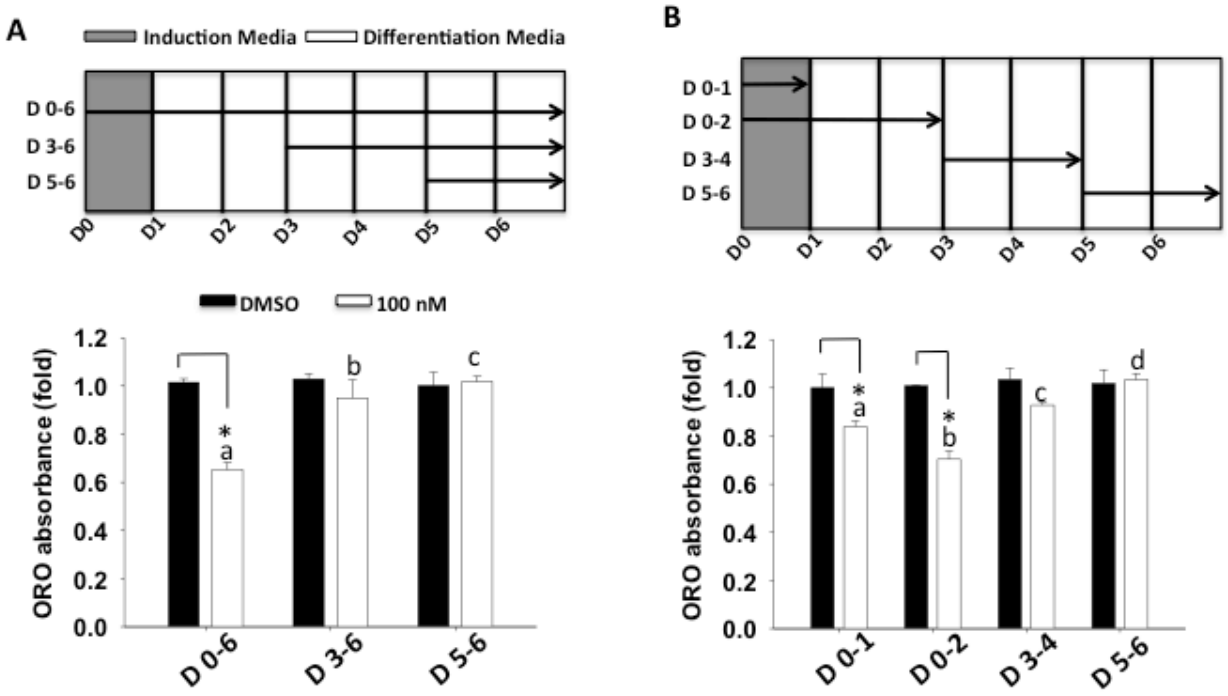

Fig. 5 Effects of 1,25(OH)$_2$D$_3$ treatment timing on brown adipocyte differentiation (A, B) Brown preadipocytes were differentiated in the presence of 1,25(OH)$_2$D$_3$ (100 nM) or the vehicle control DMSO according to the diagrams. At the end of differentiation, ORO absorbance was measured and plotted. Data are mean±SE (n=3). Different letters indicate significant difference ($p < 0.05$) among 1,25(OH)$_2$D$_3$-treated white bars. *, **, significantly different from the controls with $p<0.05$ and $p<0.01$, respectively.

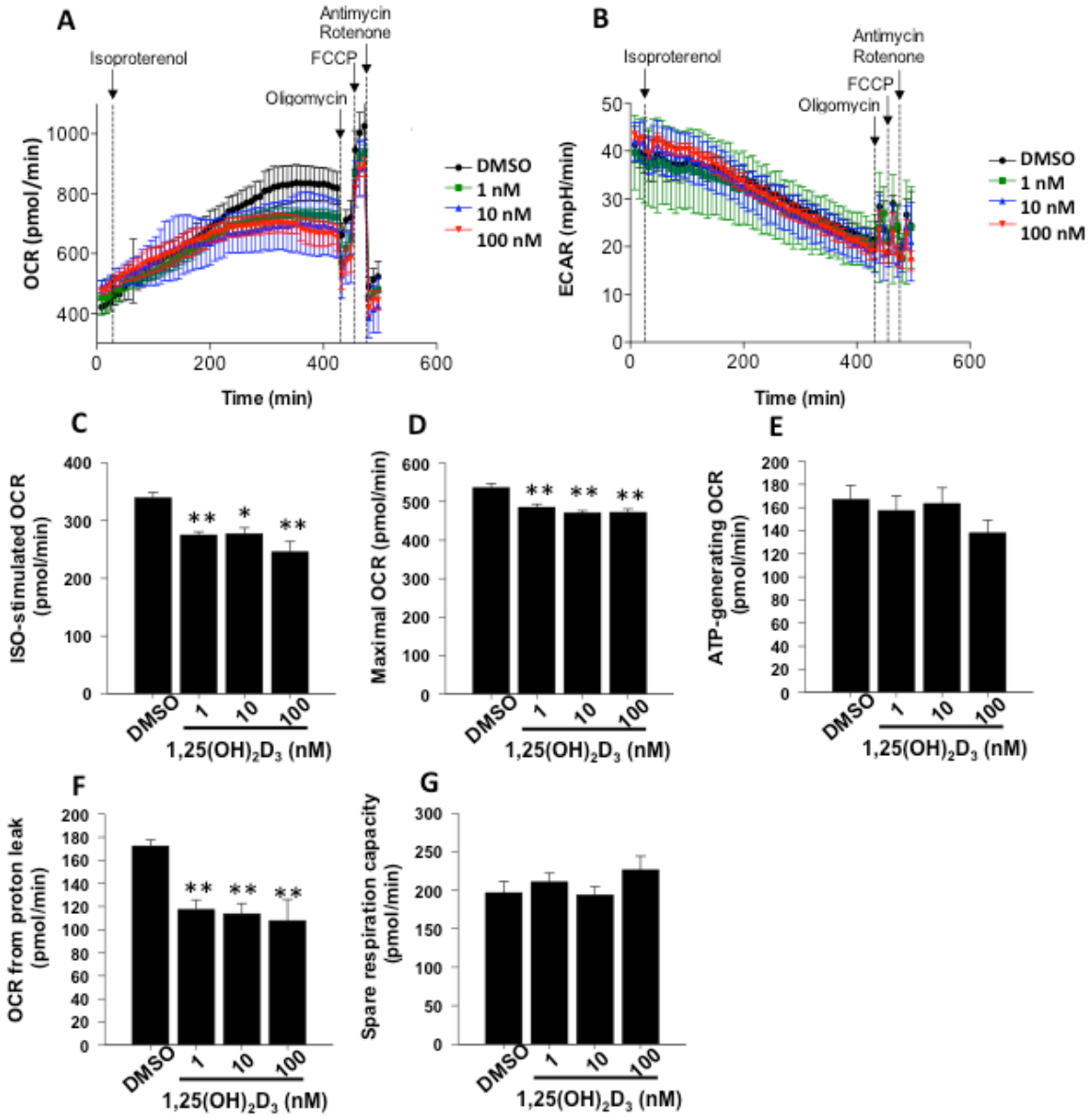

Fig. 6 Effects of 1,25(OH)$_2$D$_3$ on mitochondrial respiration in brown adipocytes
Brown preadipocytes were differentiated in the presence or absence of increasing doses of 1,25(OH)$_2$D$_3$ until day 6. The cells were then sub-cultured in 24-well XF assay plates overnight and were subjected to real-time measurements of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). Following isoproterenol (ISO, 1μM) injection, the mitochondrial complex inhibitors were injected sequentially in the following order: oligomycin (1 μM), FCCP (0.75 μM), antimycin A/rotenone (1 μM each), and the readings were taken after each inhibitor. The OCR (A) and ECAR (B) readings were plotted over time. ISO-stimulated OCR was shown in (C). Calculated maximal OCR (D), ATP-generating OCR (E), OCR from proton leak (F), Spare respiration capacity (G) were shown. Data are mean±SE (n=6-9). *, **, significantly different from the controls with $p<0.05$ and $p<0.01$, respectively.

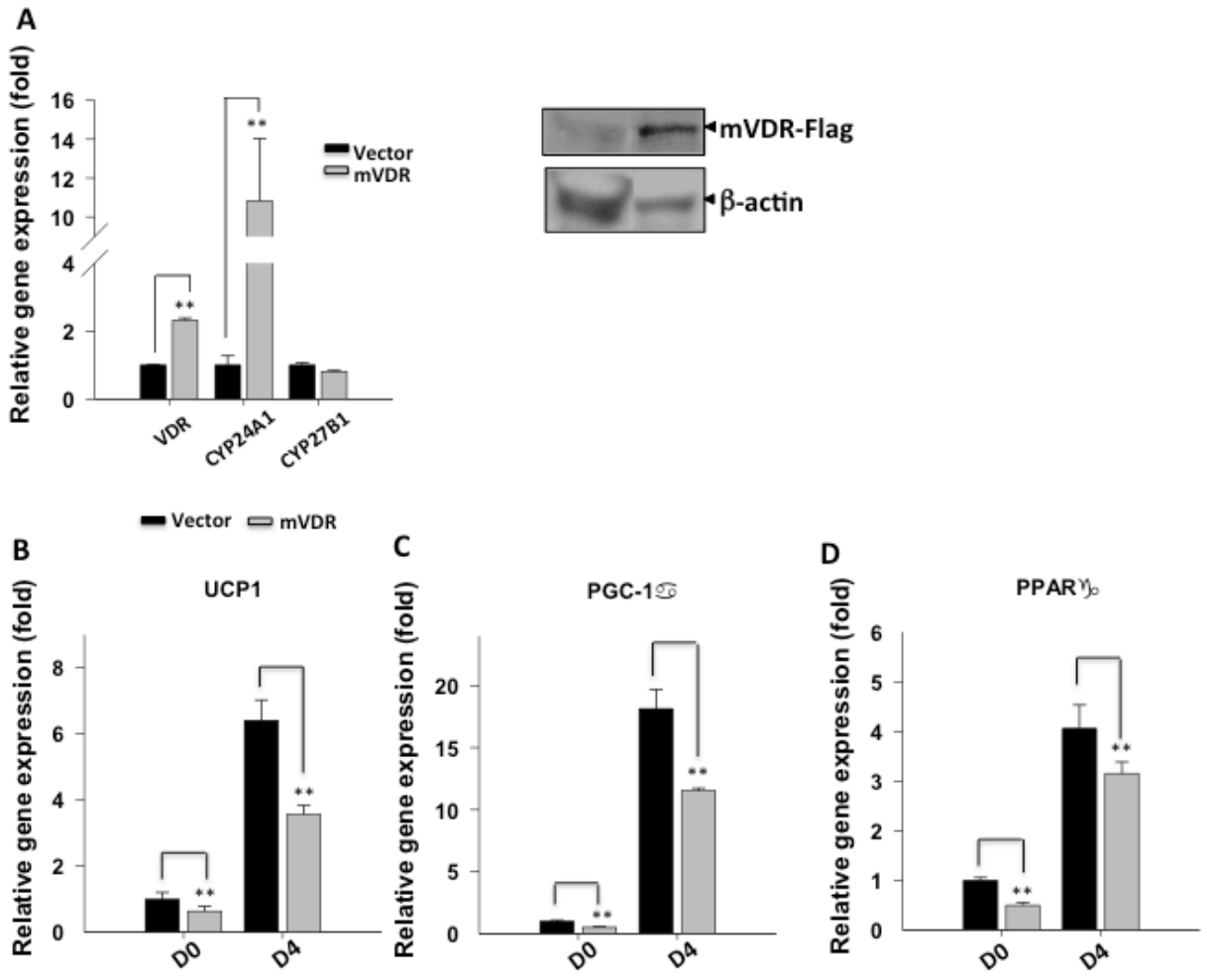

Fig. 7 Over-expression of VDR suppresses brown adipocyte differentiation
Brown preadipocytes were stably transfected with murine VDR expression plasmid, or a vector plasmid. Pools of stably transfected cells were subjected to brown adipocyte differentiation. (A) Basal mRNA expression of VDR, CYP24A1, and CYP 27B1 were shown in the left panel. The relative mRNA expression was normalized to 36B4 and expressed as fold of the vector value (set as 1). Protein expression of the Flag-tagged exogenous VDR was shown in the right panel. (B) mRNA expression of brown adipocyte marker UCP-1, PGC-1α, and PPARγ at basal (D0) and D4 in the differentiation were shown. The relative mRNA expression was normalized to 36B4 and expressed as fold of D0 value (set as 1). Data are mean±SE (n=3). *, **, significantly different from the controls with $p<0.05$ and $p<0.01$, respectively.

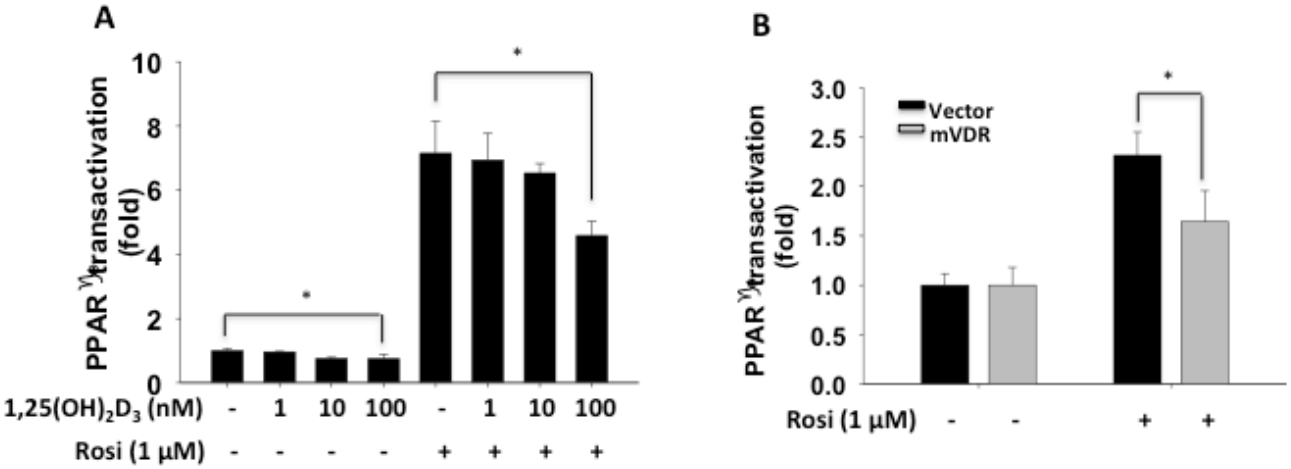

Fig. 8 1,25(OH)$_2$D$_3$/VDR suppress PPARγ transactivation in brown preadipocytes (A) Brown preadipocytes were transiently transfected with PPARγ transactivation reporters and β-galactosidase expression plasmid for 24 hr, the cells were then pre-treated with increasing doses of 1,25(OH)$_2$D$_3$ (1, 10, and 100 nM) overnight, followed by co-treatment with rosiglitazone (Rosi, 1 μM) for 8 hr. (B) Brown preadipocytes were transiently transfected with PPARγ transactivation reporters, β-galactosidase, and murine VDR expression plasmid or a vector control plasmid for 24 hr. The cells were then treated with rosiglitazone (Rosi, 1 μM) for 8 hr. Cell lysate was prepared and luciferase and β-galactosidase activities were measured. PPARγ transactivation is presented as fold of relative luciferase activities to that of the controls (set as 1). Data are mean±SE (n=3). The bars indicate dose-dependent response in (A). *, $p<0.05$.

VITA

Carolyn Jeanne Ricciardi was born to Charles Ricciardi and Jennifer Fuller-Ricciardi in Doylestown, Pennsylvania. She graduated from Central Bucks High School South in 2008. In 2012 she completed her Bachelor of Science at The Pennsylvania State University majoring in Nutritional Science and minoring in Kinesiology. During her time at Penn State she worked for the General Clinical Research Center, The Center for Childhood Obesity Research, and acted as a teaching assistant for Dr. Coleman-Kelly, RD. Upon graduation from Penn State, she was accepted into the combined Graduate Degree/Dietetic Intern program at The University of Tennessee, Knoxville. In August 2012, she began her education and candidacy for the Master's of Science in Cellular and Molecular Nutrition under Dr. Ling Zhao. She defended her thesis on June 26th, 2014. During her time at UTK, she acted as a teaching assistant for both Nutrition 100 and Nutrition 313. During her final spring and summer semesters, she also completed the dietetic internship and intends to sit for the Commission of Dietetic Registration exam.