

EXPRESSION OF VITAMIN D TARGET GENES AND PROTEINS IN
HUMAN OSTEOSARCOMA CELL LINE, 143B, IN RESPONSE TO
1 α ,25-DIHYDROXYVITAMIN D₃

by

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Submitted to the Department of Dietetics and Nutrition
and the Graduate Faculty of the University of Kansas
in partial fulfillment of the requirements for the degree of
Master's of Science

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Abstract

Osteosarcoma (OS) is the most common type of bone cancer in children and adolescents. With the current treatment approaches, the survival rate is 60-70%. Therapies for treating OS have remained the same over the past thirty years. There is a need for developing more effective therapies, which can significantly improve the survival of the patients who do not respond well to current therapeutic strategies. Epidemiological evidence indicates impaired regulation of cell proliferation and survival appears to be a key event in the etiology of the disease. Previous *in vitro* and *in vivo* studies suggest that 1 α ,25-dihydroxyvitamin D₃ (calcitriol or 1,25D) has significant antineoplastic activity by inhibiting cell proliferation and inducing differentiation and apoptosis in breast, prostate, colon, skin, and brain cancer. The molecular mechanisms for calcitriol-mediated cancer prevention are still unknown. In particular, the role of calcitriol in modulating OS needs to be investigated.

The primary objective of the study was to find out the relationship between calcitriol treatment and expression levels of vitamin D target genes: vitamin D receptor (VDR), 1 α -hydroxylase (CYP27B1), 24 hydroxylase (CYP24A1), runt related transcription factor-2 (RUNX-2), and osteocalcin (OCN), by evaluating and comparing the transcription and translation levels of the genes in response to calcitriol at 3 days, 9 days, as well as 15 days (for gene expression) and 21 days

(for protein translation) of cellular growth in OS cell line (143B). The second objective was to rule out the possibility of serum components in masking of any antiproliferative effects of calcitriol by comparing proliferation patterns of OS cell lines cultured in high serum (10%) vs. low serum (1%) and very low serum (0.1%) concentrations in OS 143B, SaOS-2, and U2OS cell lines.

Our preliminary results from this pilot study suggest calcitriol has no significant effect on expression of vitamin D target genes (VDR, CYP27B1, CYP24A1, RUNX-2 and OCN) in 143B OS cell at the mRNA and protein levels, and exerts no significant anti-proliferative effect in serum-reduced medium in 143B, SaOS-2, and U2OS OS cell lines.

Acknowledgement

I acknowledge the help of Dr. Rama Garimella as my advisor and assisted me on my research design, and thank committee for their critical review of the thesis. I appreciate Lindsey Thompson, RD for her help in the lab.

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ABBREVIATIONS

1 α ,25D ₃ -MARRS	1 α ,25(OH) ₂ D ₃ -membrane-associated rapid-response steroid binding protein
1 α ,25(OH) ₂ D ₃ or 1,25D	1-alpha, 25-dihydroxyvitamin D ₃
1 α -OHase	1 α -hydroxylase
24-OHase	24-hydroxylase
ALP	Alkaline phosphatase
CDK	Cyclin-dependent kinase
CYP24A1	Cytochrome P450, family 24, subfamily A, polypeptide 1
CYP27B1	Cytochrome P450, family 27, subfamily B, polypeptide 1
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Fetal bovine serum
HRP	Horse-radish peroxidase
MSC	Mesenchymal stem cell
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
OS	Osteosarcoma
OCN	Osteocalcin

PVDF	Polyvinylidene fluorid
qPCR	Quantitative Polymerase Chain reaction
RUNX-2	Runt related transcription factor-2
RXR	Retinoic X receptor
VDR	Vitamin D receptor
VDRE	Vitamin D response elements

Chapter 1

Introduction

Osteosarcoma (OS) is the most common type of bone cancer in children and adolescents (1). Current treatments of OS are surgery and chemotherapy. For many patients, a combination of these two treatments successfully controls the tumor. To some patients with impossible complete surgical excision and poor response to chemotherapy, a high risk of relapse and recurrence exists. Impaired regulation of cell proliferation and differentiation appears to be a key event in the etiology of the disease. Novel therapeutic regimens targeting inhibition of cell proliferation and induction of differentiation in OS are needed.

Preclinical research studies indicate calcitriol ($1\alpha,25(\text{OH})_2\text{D}_3$) exhibit potential anticancer properties due to anti-proliferative effects, activating apoptotic pathways and inhibiting angiogenesis (2). Calcitriol potentiates the anticancer effects of many cytotoxic and anti-proliferative anti-cancer agents. One possible anti-proliferative mechanism of calcitriol in OS cells is binding to a nuclear vitamin D receptor (VDR), which acts as a transcription factor and modulates the expression of genes involved in cell cycle progression (3, 34-38), differentiation (4), angiogenesis (2, 6), and apoptosis (6, 40-44).

Calcitriol induces differentiation in numerous types of benign and malignant cells (4). Activation of transcription factors by calcitriol increases expression of

multiple genes involved in differentiation. Emerging evidence suggests osteosarcoma should be considered as a differentiation disease with defects in osteoblast differentiation from mesenchymal stem cells (MSC) (5). Therapies of promoting differentiation in OS cells can be an effective treatment strategy against OS.

Calcitriol modulates the expression of pro- (Bax, Bak and Bad) and anti-apoptotic (Bcl-2) in breast, colon, prostate, myeloma, and lymphocytic leukemia cells (6).

In vitro and in vivo studies clearly demonstrate that calcitriol modulates proliferation, differentiation and apoptosis of tumor cells. The mechanisms underlying the antineoplastic effects of calcitriol remain to be determined.

Statement of Purpose

Previous studies in Dr. Garimella's lab demonstrated that both 25(OH)D₃ and calcitriol can stimulate differentiation and apoptosis, however, neither one has any significant effect on proliferation of two OS cell lines, 143B and SaOS-2. The exact mechanism(s) underlying calcitriol induced differentiation and apoptosis is (are) not clear.

In order to find out the role of vitamin D in OS cells in proliferative and differentiative stages and the transition between these two stages, the present study investigated vitamin D target genes (including VDR, CYP27B1, CYP24A1,

RUNX-2, and osteocalcin-OCN) by comparing the expression of these genes in response to calcitriol during proliferation, post-proliferation, and differentiation of OS cell lines, 143B versus control osteoblast (Ob, MC3T3-E1-mouse pre-osteoblast cell line).

To rule out the possibility of activity of vitamin D binding serum proteins that may play a role in suppressing or masking the antiproliferative potential of vitamin D against osteosarcoma, we compared the proliferation patterns of OS cell lines in high serum vs. low serum growth conditions.

In this project, the osteosarcoma cell line, 143B, was evaluated for vitamin D target gene (VDR, CYP27B1, CYP24A1, RUNX-2 and OCN) in response to calcitriol treatment. The 143B cell line has high metastatic potential and harbors a mutation in *Ras* gene. Activation of *Ras* gene is commonly seen in colon cancer and pancreatic cancer. Clinical significance of occurrence of *Ras* activation in OS is not clear. Not many studies have been done in 143B cell line until now. This osteosarcoma cell line is of interest to study.

Study Objective

The primary objective of this study is to find out the relationship between calcitriol treatment and expression levels of vitamin D target genes by evaluating and comparing the expression and translation levels of the genes in response to calcitriol at 3 days (defined as proliferation stage), 9 days (defined as

post-proliferation stage), 15 days (defined as differentiation stage for gene expression), and 21 days (defined as differentiation stage for protein translation) of cellular growth in OS cell line (143B).

The secondary objective of this study was to rule out the possibility of serum components in masking of any antiproliferative effects of calcitriol by comparing proliferation patterns of OS cell lines cultured in high serum (10%) vs. low serum (1%) and very low serum (0.1%) concentrations.

Chapter 2

Literature Review

Osteosarcoma

Osteosarcoma is a malignant bone tumor predominant among children and adolescents. About 400-600 cases of OS are reported annually. Using the current treatment options, the patients with osteosarcoma have a 60-70% survival rate. The disease is severe in the remaining one third of the population who does not respond well to chemotherapy and often succumb to pulmonary metastasis. There is a need to identify novel therapeutic regimens against osteosarcoma.

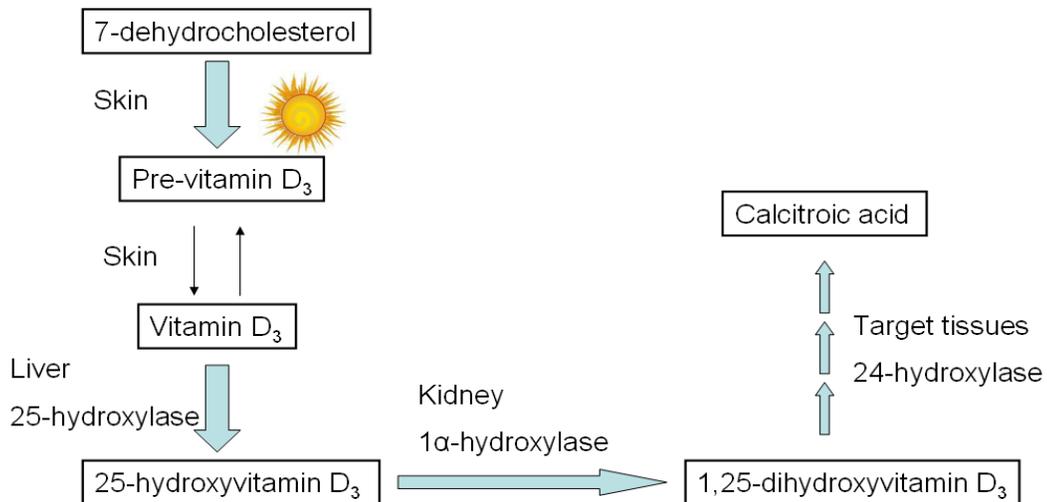


Figure 1 Vitamin D Metabolism. In response to UV light, 7-dehydrocholesterol and pre-vitamin D₃ convert to vitamin D₃. Vitamin D₃ transforms to 25-hydroxyvitamin D₃ in the liver by 25-hydroxylase. In the kidney, 25-hydroxyvitamin D₃ undergoes a hydroxylation reaction in the presence of 1 α -hydroxylase to form 1,25-dihydroxyvitamin D₃. Calcitriol is catabolized by 24-hydroxylase to calcitroic acid.

Vitamin D is a secosteroid hormone historically known for its role in maintaining bone health by regulating calcium and phosphate homeostasis. The chemical structures of vitamin Ds were first specified in the 1930s (64). The classic bone disease resulting from a deficiency in vitamin D is called rickets in children and osteomalacia in adults. Vitamin D comes in many forms. The two most important isomers are vitamin D₂ and vitamin D₃. Vitamin D₂ comes from yeasts and plants while vitamin D₃ is rich in fatty fish and fatty fish oil. Vitamin D can be obtained from fortified foods such as milk and milk products. It also can be synthesized in skin in the presence of UV light (Figure 1). Exposure of the skin to the UV rays of sunlight induces the conversion of 7-dehydrocholesterol to previtamin D₃. Then previtamin D₃ undergoes a thermal isomerization to vitamin D₃. Vitamin D₃ is transported to the liver where it is converted to 25-hydroxyvitamin D₃ or 25(OH)D₃, the major circulating metabolite of vitamin D₃ in the body. The final activation in the kidney is catalyzed by 1 α -hydroxylase (1 α -OHase) forming calcitriol (1 α ,25(OH)₂D₃), the active form of the vitamin. Catabolic inactivation is carried out by 24-hydroxylase (24-OHase).

Calcitriol, the biologically active form of vitamin D, is historically known for its role in regulating calcium and phosphorus homeostasis. Epidemiological studies point to a relationship between vitamin D deficiency and cancer risk. The role of calcitriol in the control and regulation of cellular growth and differentiation is emerging in various cell types, including carcinomas of the breast, prostate, colon,

skin, and brain, myeloid leukemia cells, and others (11,12), and of significant interest to cancer researchers.

Calcitriol has significant antineoplastic activity in numerous *in vivo* and *in vitro* studies suggesting the hypothesis of several mechanisms involving in the control of the activity. The mechanisms underlying the antineoplastic roles of $1\alpha,25(\text{OH})_2\text{D}_3$ are not clear and they may vary with different tumor models and experimental conditions. Calcitriol executes its anticancer effect by inhibiting proliferation associated with cell cycle arrest (3, 34-38), inducing differentiation (4) and apoptosis (6, 40-44) and by reducing invasiveness as well as angiogenesis (2, 6).

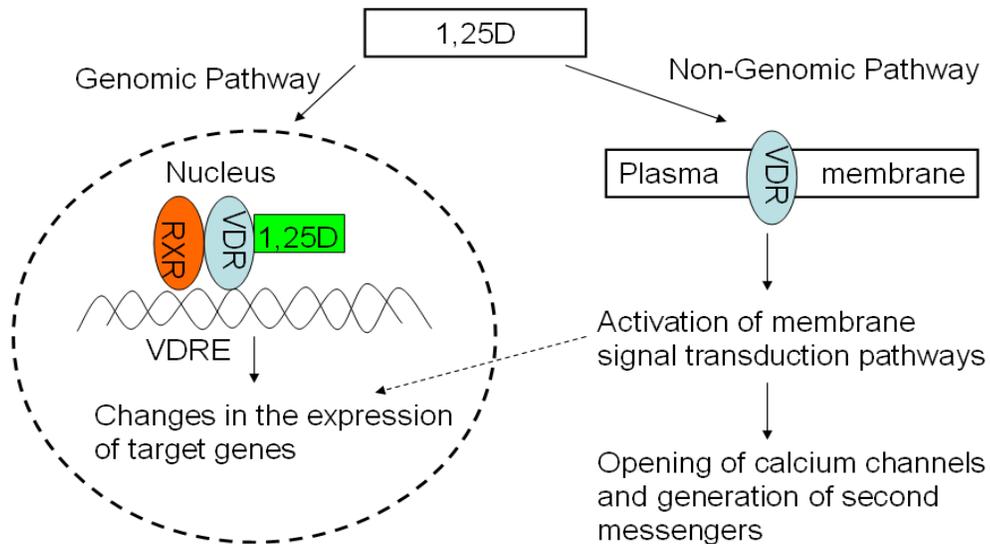


Figure 2 Genomic and non-genomic pathways of calcitriol mode of action. In the genomic pathway, calcitriol binds to the VDR and recruits retinoic X receptor (RXR) to form a complex which interacts with vitamin D response elements (VDRE) to regulate gene transcriptions. In non-genomic pathway, calcitriol binds to plasma membrane VDR to execute rapid effects. (Source: Rama Garimella)

Calcitriol exerts its antineoplastic activity through both genomic and non-genomic pathways (Figure 2). The genomic pathway is mediated by the binding of calcitriol to VDR. This binding activates VDR to recruit cofactors, including retinoic acid X receptor (RXR), to form a complex to bind to vitamin D response elements (VDRE) located in the promoter region of target genes and regulate gene transcriptions (13). VDR is a member of the steroid hormone receptor superfamily and is present in many tissues, including intestine, kidney, bone, brain, stomach, heart, pancreas, skin, activated T and B lymphocytes, colon, ovary, breast, and prostate (14,15). It regulates gene expression in a ligand-dependent manner (16). Multiple genes contain VDRE in their promoter region, including bone-related genes (6). These findings suggest that vitamin D play a role in controlling cellular growth in the non-neoplastic normal tissues. However, VDR is not the only factor responsible for the efficacy of calcitriol in controlling tumor growth. In prostate cancer cells, altered expression of the steroid receptor corepressor SMRT or defective VDR localization determined the resistance to antiproliferative effects of calcitriol (17).

Nongenomic actions of calcitriol are rapid and do not depend on transcription directly. This pathway begins at the plasma membrane and involves a non-classical membrane receptor (18) and a $1\alpha,25(\text{OH})_2\text{D}_3$ -membrane-associated rapid-response steroid binding protein ($1\alpha,25\text{D}_3$ -MARRS) (19). Nongenomic effects of calcitriol can activate the

Raf-mitogen-activated protein kinase extracellular signal-regulated kinase kinase (MEK)-mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK) cascade in skeletal muscle cells (20), which subsequently increases the transcriptional activity of the VDR and stabilizes the receptor, thereby cooperates with the classical genomic pathway to activate VDR and lead to the antiproliferative effects of calcitriol.

Abe et al. for the first time reported that calcitriol stimulates terminal differentiation in mouse myeloid leukemia cells, thereby suggesting an antineoplastic role for vitamin D against myeloid leukemia (21). Since then many *in vivo* and *in vitro* studies have demonstrated the anticancer effects of calcitriol in the cancer of bladder (22), breast (23), colon (24), endometrium (25), kidney (26), lung (27), pancreas (28), prostate (29), osteosarcoma (30), neuroblastoma (31), glioma (32), melanoma (33), and others. The anticancer effect of calcitriol is mainly through the enhancement or suppression of the transcription of key genes by the ligand-activated VDR. Indirectly, vitamin D also can alter cellular growth by interacting with other important transcriptional factors or cell signaling pathways affecting on cell cycle progression (34-38), differentiation (4), and apoptosis (6, 40-44).

Calcitriol exerts its antineoplastic effects mainly by antiproliferative actions. Calcitriol induces arrest in the cell cycle in numerous cell lines (24-38). Cell cycle consists of 5 phases including gap 0 (G0), gap 1 (G1), synthesis (S), gap 2 (G2),

and mitosis (M) (Figure 3). The major arrest is at the G0/1 to S phase transition. Calcitriol activates the transcriptions of cyclin-dependent kinase (CDK) inhibitors p27^{Kip1} and p21^{Waf1} while CDK is important for the transcription of many genes necessary for DNA replication, mitosis, and control of phase transition during cell cycle. The activation of CDK inhibitors is responsible for cell cycle arrest (34, 35).

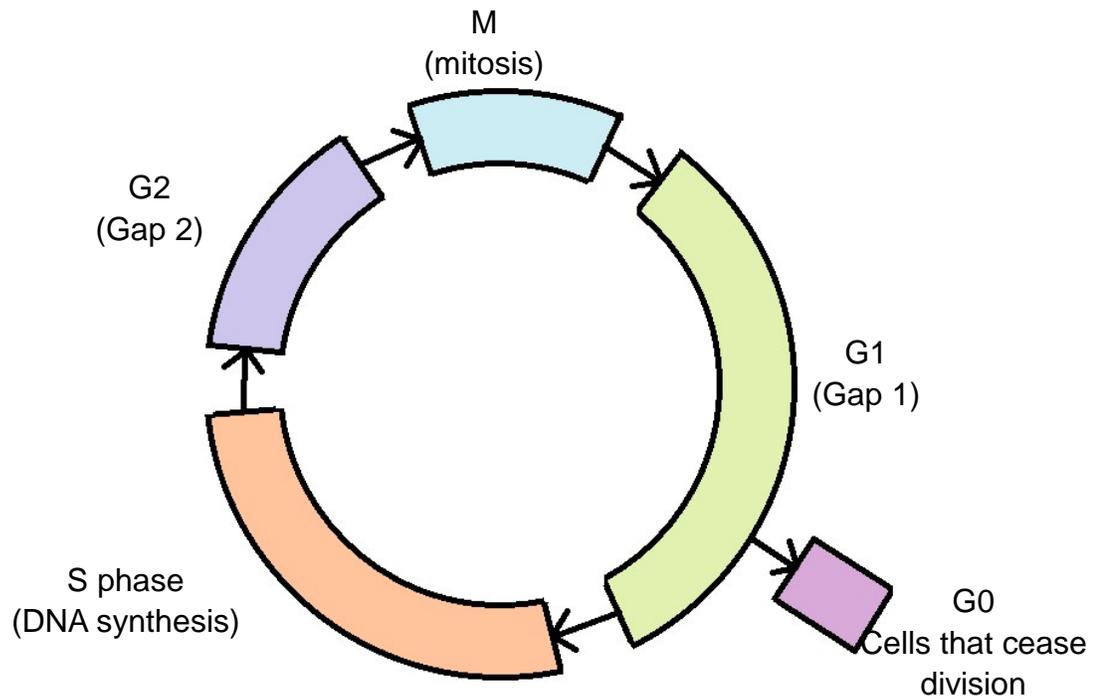


Figure 3 Cell Cycle. Cell cycle consists of 5 phases including gap 0 (G0), gap 1 (G1), synthesis (S), gap 2 (G2), and mitosis (M).

Besides the CDK inhibitors, mitogenic signals may also be regulated by calcitriol, including signaling through the mitogenic ERK/mitogen-activated protein kinase pathway, to inhibit cellular growth (44). In addition, calcitriol elicits antiproliferative effects by interfering with growth factor signaling such as the

epidermal growth factor receptors (45), transforming growth factor β (46, 47), and components of the insulin-like growth factor system (48-50).

Vitamin D compounds may alter cellular proliferation variably in different cell types. Calcitriol and vitamin D analogues had an antiproliferative effect on the growth of primary cultures of human prostatic cells (51). In contrast, in normal mouse epidermis calcitriol induces cell proliferation (53). Concentrations of calcitriol used in cell culture studies also are critical in modulating effects on cellular proliferation and differentiation. In primary mouse epidermal keratinocytes, low concentration (picomolar) of calcitriol stimulates proliferation while high concentration (nanomolar to micromolar) inhibits proliferation (53). The timing of induction of antiproliferative effect of calcitriol varies from cell to cell and also dependent on dose or concentration calcitriol. More research is needed to clarify the underlying mechanisms of cell- and dose-specification.

Calcitriol induces differentiation in numerous types of benign and malignant cells (4). Calcitriol induces differentiation by activation of transcription factors leading to expression of multiple genes involved in differentiation. Work conducted at Dr. Garimella's lab had shown higher concentration (≥ 100 nM) of calcitriol increased alkaline phosphatase (ALP) enzyme activity in osteosarcoma 143B cell line suggesting vitamin D may act as a differentiation agent in these cells.

According to emerging evidence, osteosarcoma is commonly regarded as a

disease caused by genetic and epigenetic disruptions of osteoblast differentiation pathways from mesenchymal stem cells (MSC) (5). Osteogenic stimuli failed to induce differentiation of most OS cells which indicates the possible differentiation defects exist in OS cells (55). Promoting differentiation in otherwise uncontrolled rapidly proliferating OS cells is a potential therapy for OS in contrast to current chemotherapies focus on reducing proliferation of OS tumors.

Induction of apoptosis also plays a key role in anticancer effect by calcitriol in the breast cancer (39), colon cancer (40), prostate cancer (41), myeloma (44), and lymphocytic leukemia (42). But vitamin D exerts no effects on induction of apoptosis in normal astrocytes, melanocytes, and mammary cells (56). In MCF-7 breast cancer cells (39), prostate cancer cells (41) and B-cell chronic lymphocytic leukemia cells (43), calcitriol down-regulated the anti-apoptotic protein Bcl-2 which is overexpressed in many cancer cells. In some cancer cell lines, calcitriol also induced up-regulation of the pro-apoptotic protein Bax. The stimulation was independent on p53. Calcitriol induces apoptosis by tipping the balance of pro-apoptotic and/or anti-apoptotic proteins toward apoptosis rather than cell survival (4).

Markers of cellular responsiveness to the antineoplastic action of calcitriol

Antineoplastic effects of calcitriol are variable in different cell types, even the cells derive from the same tissue. Genes regulating vitamin D metabolism (VDR,

CYP27B1, and CYP24A1) and osteoblastic differentiation (RUNX-2 and OCN) genes are regarded as good markers to determine the cellular responsiveness of OS cell lines to calcitriol treatments.

Vitamin D Receptor (VDR)

There is enough evidence regarding a positive correlation between the status of VDR and the effects of calcitriol. To some extent, the expression level and the nuclear localization of the VDR determine the cellular sensitivity to growth inhibition of calcitriol. Studies in VDR knockout models demonstrated the significance of calcitriol/VDR interaction and signaling pathways in response to and execution of the anticancer activity of calcitriol. For example, VDR-null mice showed hyperproliferation and increased mitotic activity in the descending colon (56). VDR ablation in mouse increased chemical carcinogenesis in mammary, epidermis and lymphoid tissues (57).

1 α -Hydroxylase (1 α -OHase) and 24-Hydroxylase (24-OHase)

The final activation of calcitriol from 25(OH)D₃ occurs in the kidney and is catalyzed by the enzyme 1 α -OHase (encoded by gene CYP27B1). The catabolic degradation is carried out by the enzyme 24-OHase (encoded by gene CYP24A1). Calcitriol is tightly controlled by the feedback regulation in the cells to prevent toxicity of vitamin D. Excess build-up of calcitriol is prevented by the cooperative

actions of 1α -OHase and 24-OHase. Thus, expression levels of these two enzymes may indicate the metabolism status and/or activity of calcitriol in the cells.

RUNX-2

RUNX-2 is a member of the runt-related transcription factors and an essential mediator of the osteoblast phenotype and plays a critical role in the process of osteoblast differentiation. RUNX-2 has suppressive activity in osteoblasts and is controlling osteoblast differentiation (65). RUNX-2-null model demonstrated a cartilaginous skeleton with complete absence of ossification suggesting the importance of RUNX-2 in chondrogenesis and osteogenesis (53). In addition, when RUNX-2 is overexpressed in chondrocytes, it causes ectopic chondrocyte hypertrophy and endochondral ossification, suggesting the importance of RUNX-2 in controlling differentiation of both chondrocytes and osteoblasts (5). RUNX-2 is expressed at higher levels in human osteosarcoma cell line OS1 than in human fetal osteoblasts (65). The expression of RUNX-2 gene is a common and appropriate marker for osteogenic differentiation.

Osteocalcin (OCN)

Osteocalcin (OCN) is a noncollagenous protein found in bone. It is secreted by osteoblasts and plays an important role in regulating bone mineralization as

well as calcium homeostasis. Mineralization of extracellular matrix is a characteristic feature of a differentiated osteoblast. Osteocalcin is often used as a biochemical marker or biomarker for the bone mineralization process.

Altered expressions and functional activities of proteins of vitamin D target genes (VDR, CYP27B1, CYP24A1, RUNX-2 and OCN) are observed in many tumor types (2). There is a need to clarify the expression levels of these markers in OS cells.

Role of fetal bovine serum (FBS) in modulating calcitriol mediated effects on cellular proliferation and differentiation

Fetal bovine serum is an undefined reagent to provide growth factors for cell culture. Most investigators considered the components in FBS would mask or suppress the antiproliferative effect of calcitriol. Thereby, to reduce the variable and uncertain factors, multiple studies (58-61) grow cells in serum-reduced medium or other defined growth factors or use of charcoal-stripped serum. Calcitriol has biphasic effects on cell growth with/without the presence of a growth medium containing whole FBS in prostate cancer cells (66). Some prostate cancer cell lines are inhibited by calcitriol in the presence of 10% FBS, however, are stimulated in the presence of charcoal-stripped, sulfatase-treated FBS suggesting calcitriol might interact with factors in FBS (66).

Chapter 3

Methods

Setting

Experiments were conducted at The University of Kansas Medical Center (KUMC) in the Bone Research Lab (G002 WHE) and G003 Lied Building and the department of Dietetics and Nutrition from January, 2009 to April, 2010.

Ethics

This study qualifies for exemption from Human Subjects Approval as the cell lines used in the proposed study are considered Secondary data. The cells are publicly available and contain no identification markers.

Materials and Methods

Reagents

Calcitriol was purchased from Enzo Life Sciences International Inc., (Plymouth Meeting, PA). Stock solutions were prepared and frozen at -20°C. Mito+ serum extender were purchased from Thermo Fish Scientific Inc., (Waltham, MA) and used according to manufacturer's recommendation.

Cell Lines

Human osteosarcoma cell lines, 143B (purchased from American Type Culture Collection, Rockwell, MD) and control osteoblast cells (MC3T3-E1-mouse pre-osteoblast cell line) (provided by Professor Rowe from Kidney Institute, University of Kansas Medical Center, Kansas City, Kansas) were used in this study.

Stock Cell Culture

Osteosarcoma cells and osteoblast cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% and 20% fetal bovine serum (FBS), respectively, as stock cultures. Cells were maintained by standard methodology at 37°C in humidified (95% air : 5% CO₂) incubator.

Cell Culture for Experiments

For different experiments, cells were seeded in 6 well plates in DMEM supplemented with 1% FBS and 0.1% mito+ serum extender. The standard cell density of 143B and MC3T3 were 0.5×10^4 and per well, respectively. Mito+ serum extender is a concentrated, fully defined formulation of hormones, growth factors (EGF and FGF), and other metabolites (insulin and steroid hormones). The use of mito+ serum extender instead of FBS for cell growth is to rule out the undefined factors (vitamin D binding proteins present in the serum) masking the

effects of vitamin D.

Proliferation and Post-proliferation Study

Osteosarcoma and osteoblast cells were plated in 6 well plates with the standard cell density. Ethanol (0.01%) (control) or calcitriol (100 nM) were added to the cultures 4 h after plating. Medium was changed every two days with fresh treatments (ethanol control and calcitriol) (Figure 4). Based on previous study in Dr. Garimella's lab, the initiation concentration of antineoplastic effects of calcitriol is 100 nM. In this study, we used 100 nM calcitriol as treatment.

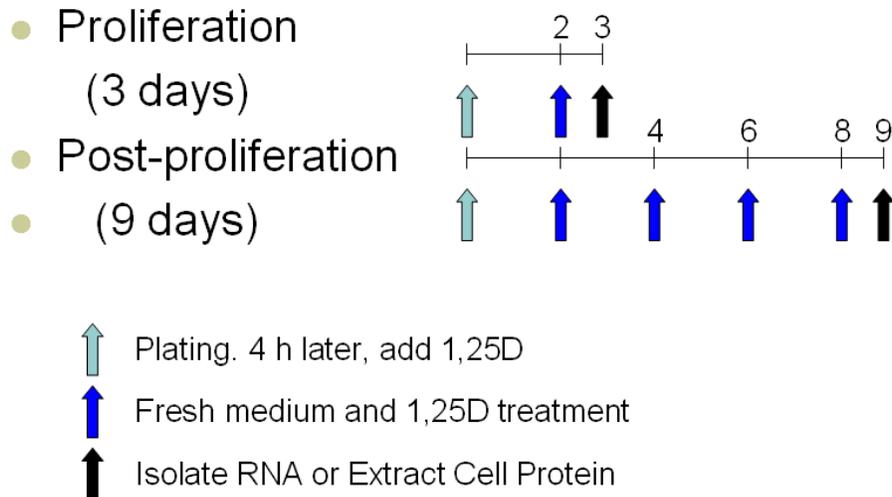


Figure 4 Schedules of Proliferation and Post-Proliferation Studies. Arrows indicate treatment/processing protocol.

After 72 h (proliferative) or 9 days (post-proliferative), RNA was isolated by using RNeasy Mini Kit (Qiagen, Santa Clara, CA) and cell-lysates containing

proteins were prepared by lysing the cells in Triton-lysis buffer. For each experiment, N equals to or is larger than three.

- Realtime qPCR (15 days)
- Western Blot (21 days)

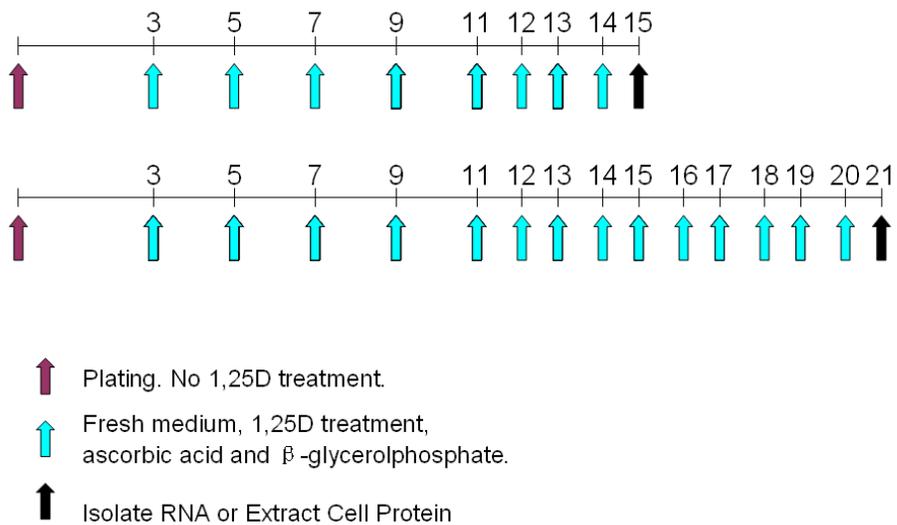


Figure 5 Schedule of Differentiation Study. Arrows indicate treatment/processing protocol.

Differentiation Study

Osteosarcoma and osteoblast cells were plated in 6 well plates with the standard cell density. Ethanol (0.01%) (control) or calcitriol (100 nM) were added to the cultures 3 days after plating. Differentiation-inducing agents, L-ascorbic acid 2-phosphate and β -glycerophosphate, were added at the concentrations of 50 μ g/ml (67) and 5 nM, respectively. Medium was changed every two days with fresh treatments.

After 15 days, RNA was isolated by using RNeasy Mini Kit (Qiagen, Santa

Clara, CA) while after 21 days, protein was extracted by using Triton-lysis buffer (Figure 5). For each experiment, N equals to or is larger than three.

Real-time Quantitative Polymerase Chain reaction (qPCR)

RNA was reverse-transcribed by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Silicon Valley, CA). Complementary DNA was tested with probing VDR, CYP27B1, CYP24, Runx2 and OCN by real-time qPCR. All primers were tested and the optimal primer concentrations were determined at 900 nM. Real-time qPCR was performed using 7500 real time PCR system (Dr. Jinxi Wang, Department of Orthopedic Surgery, University of Kansas Medical center) according to the standard protocol as recommended by Applied Biosystems 7500 sequence Detection system and software (Applied Biosystems, Foster City, CA). Sequences of all PCR primers are published by Atkins et al. in 2007(62). Relative expression between samples was calculated using the comparative cycle threshold (C_T) method (ΔC_T) (62). The formula is $X_N = 2^{-\Delta C_T}$. X_N is the relative amount of target gene and ΔC_T is the difference between the C_T of the gene and the C_T for the GAPDH. The calibrator, MC3T3E1, was used in this study.

Western Blotting

The expression of VDR, 1 α -OHase, 24-OHase, RUNX-2, and OCN proteins

were assessed by western blotting analysis. Protein concentration of the crude cell lysates was determined by Bio-Rad DC protein assay. About 40-60 µg protein of crude cell lysate was solubilized in SDS-sample buffer, and electrophoresed on a 12% denaturing polyacrylamide gel. For western blotting, the proteins from the gel were transferred on to a polyvinylidene fluoride (PVDF) microporous membrane and incubated with the primary antibodies: anti-VDR, anti-1 α -OHase, anti-24-OHase, anti-Runx2, and anti-OCN. The concentration of all primary antibodies is 200 µg/ml. The working concentration of the above primary antibodies used in western blot analysis is 1:200. The primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Following incubation with horse-radish peroxidase (HRP) conjugated secondary antibody, the immunostained bands were visualized by ECL chemiluminescent detection system (Amersham Biosciences, Piscataway, NJ). The developed films were scanned using a desktop scanner (HP ScanJet 4100C) and final images of western analyses were created using Adobe Photoshop. Breast cancer cell MCF7 cell extract and kidney tissue extract are used as positive control.

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) Cell Proliferation Assay

Cells were cultured in 96 well plates in DMEM containing 1% and 0.1% FBS. The standard cell density of 143B cell line was 8000 cells per well. Ethanol

(0.01%) (control) or calcitriol (10 nM and 100 nM) were added after 4 h and 48 h.

After 96 h, cellular proliferation was assayed by MTS assay.

Analysis of Data

Excel software (Microsoft Corp., Redmond, WA, USA) was used for all statistical analysis. Means and standard deviations were calculated for all data measures. T-test and Analysis of Variance (ANOVA) was used to analyze differences in experimental and control data, and differences between groups. A p value < 0.05 was considered statistically significant.

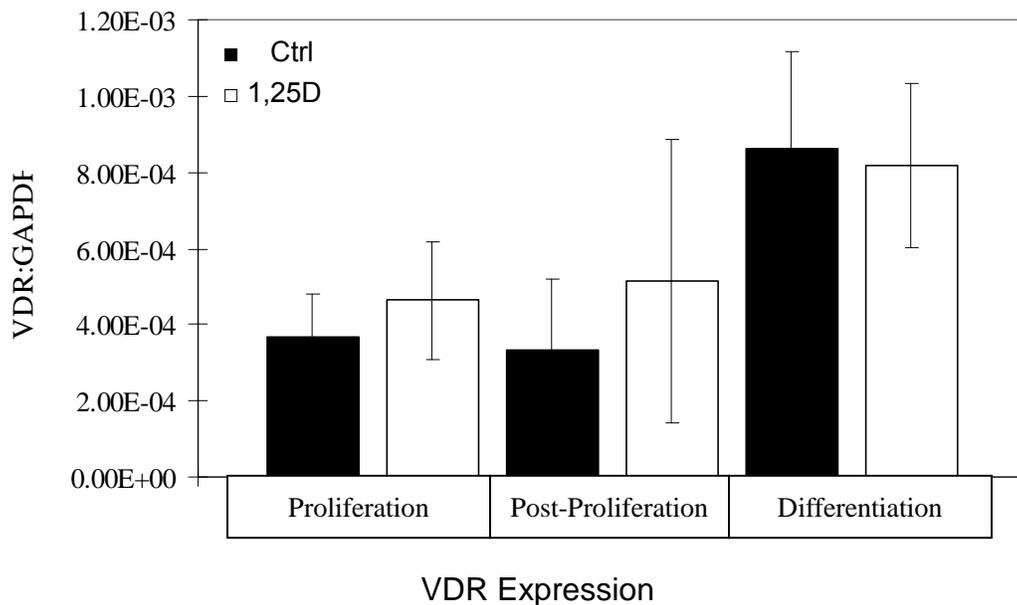
Chapter 4

Results

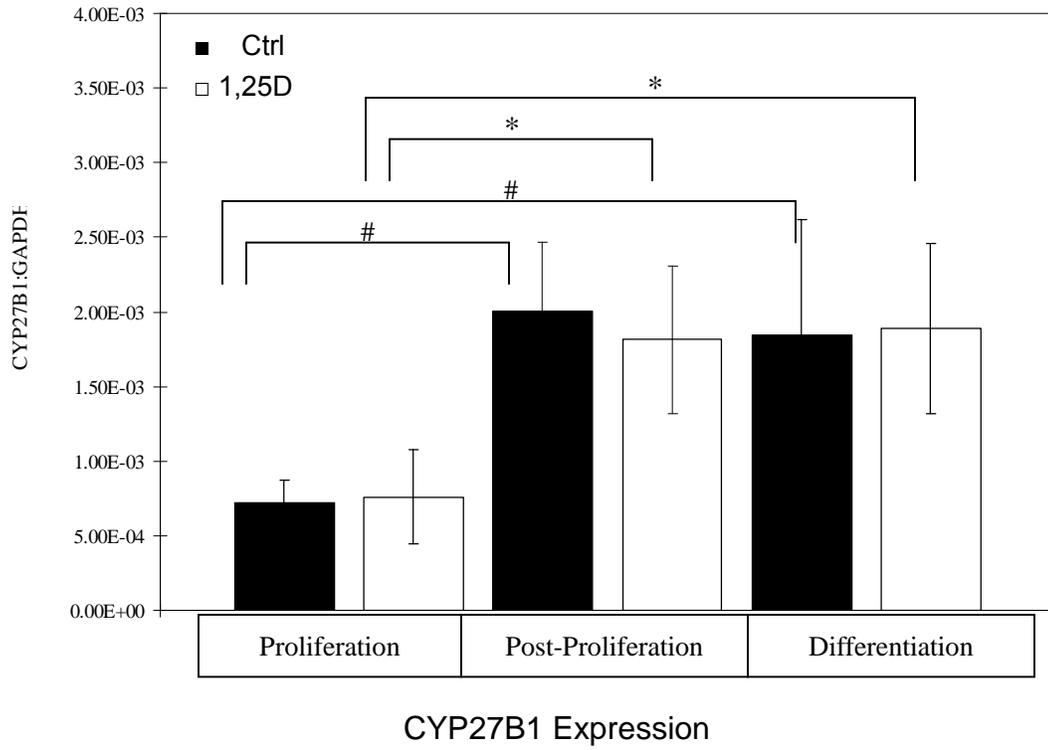
Expression of Vitamin D Target Genes mRNA in Osteosarcoma 143B

Vitamin D target genes (VDR, CYP27B1, CYP24A1, RUNX-2, and OCN) are expressed in 143B OS cells. No significant differences exist between control and calcitriol-treated groups in proliferative, post-proliferative, and differentiation stages. No significant differences of VDR, CYP24A1, RUNX-2, and OCN are found when comparing control or calcitriol-treated samples in three stages. Only CYP27B1 mRNA is increased in post-proliferative and differentiation stages when comparing to proliferative stage. Expression of OCN mRNA is very low comparing to the other genes.

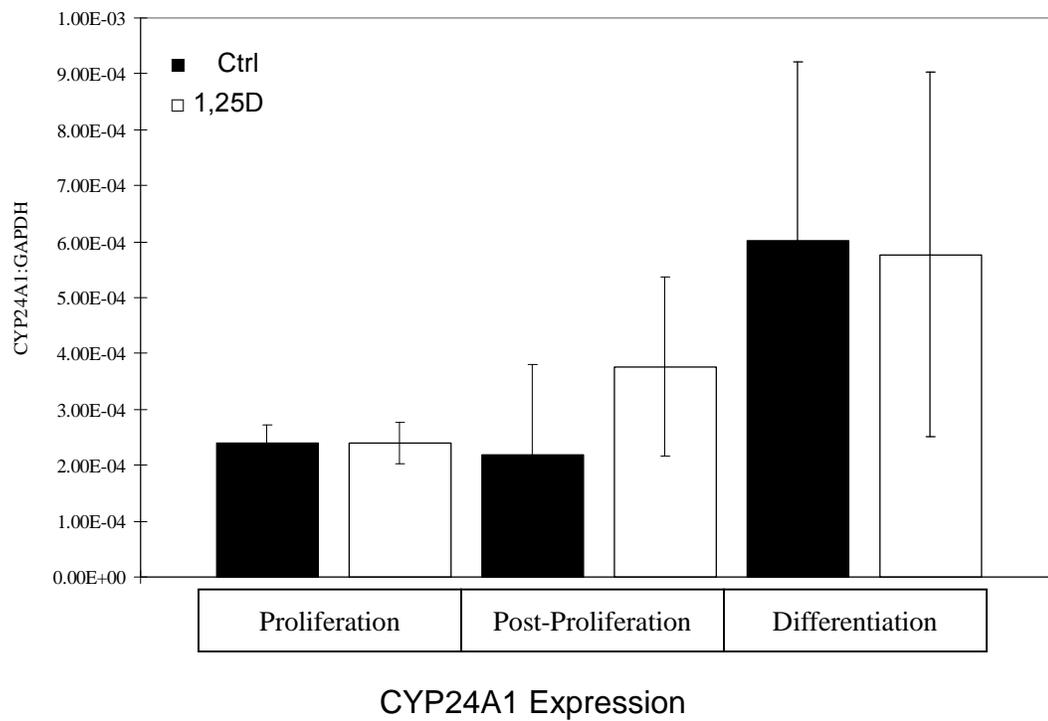
A.



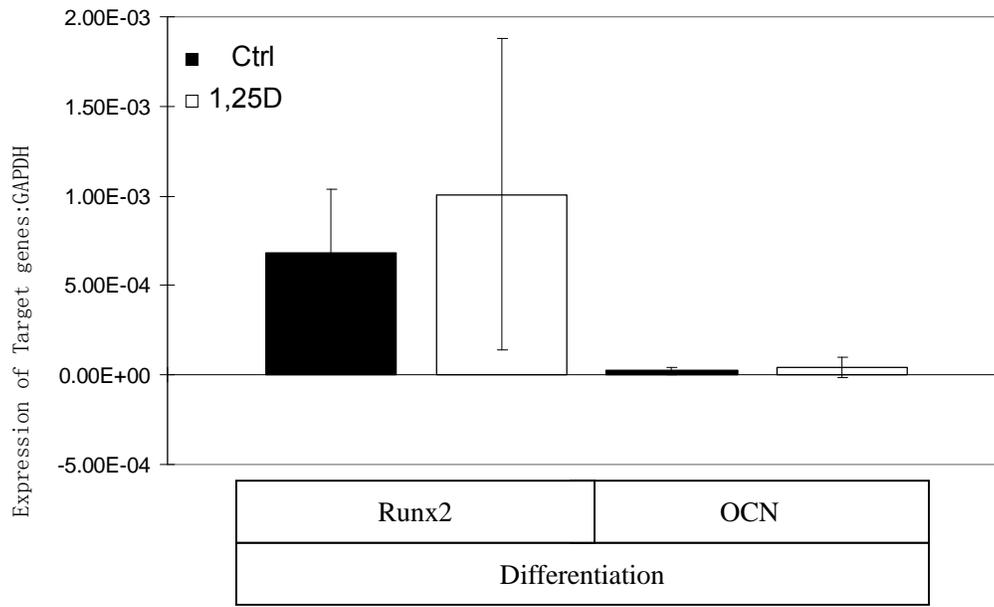
B.



C.



D.



RUNX-2 & OCN Expression

E.

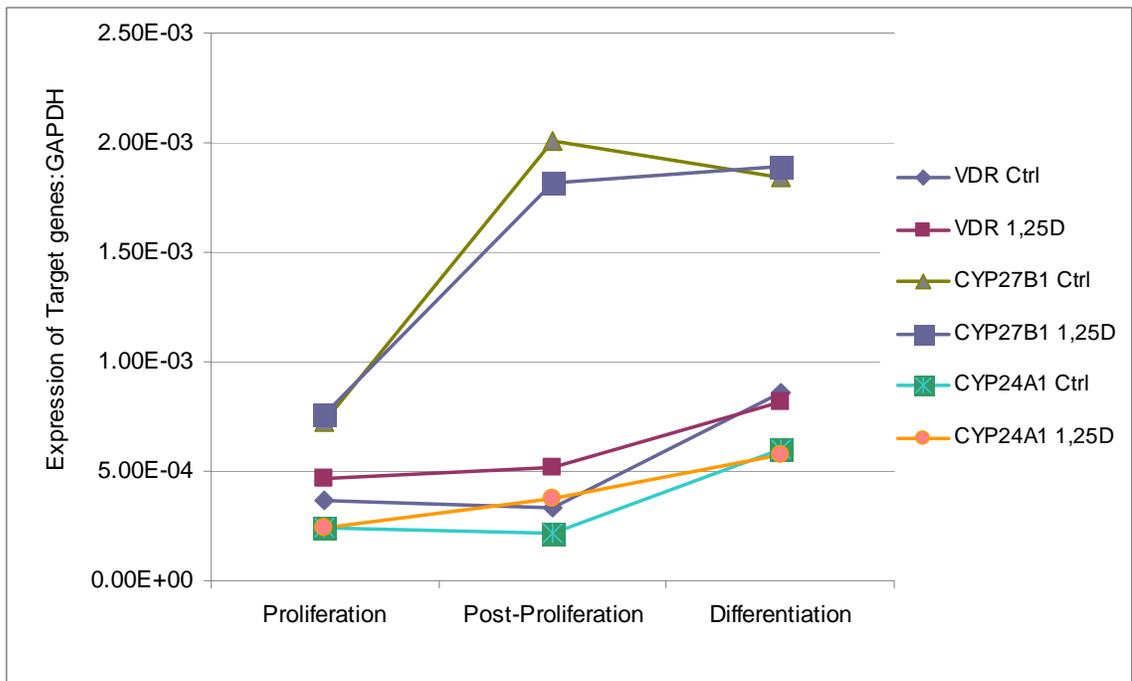


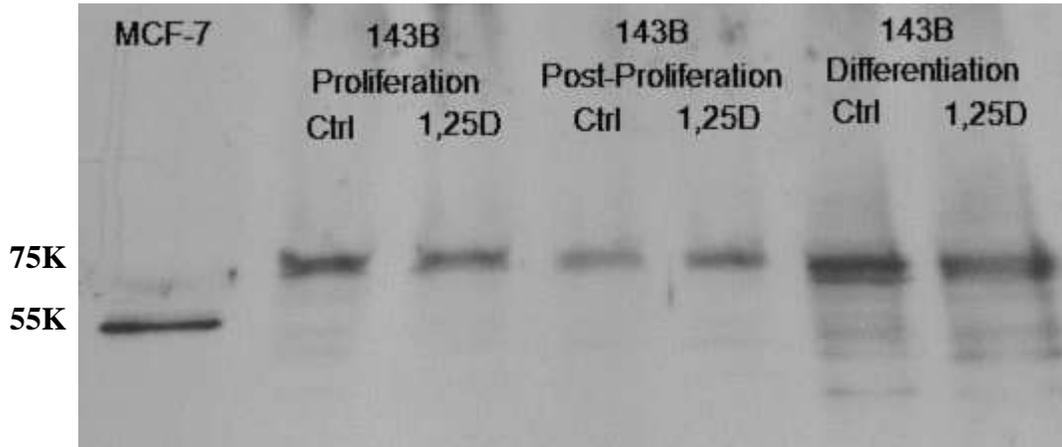
Figure 6. Effect of calcitriol on vitamin D target gene expressions. 143B was cultured in serum-reduced medium (supplemented with mito+ serum extender), with or without the

addition of calcitriol (100 nM) for 3 days, 9 days, and 15 days, and processed for real-time PCR analysis, for (A) VDR, (B) CYP27B1, (C) CYP24A1, (D) RUNX-2 and OCN mRNA expression, as described. Data are expressed as mean expression normalized to GAPDH, \pm S.D. of triplicate reactions performed on at least triplicate samples. Significant difference was determined using Student's t-test and ANOVA, and is indicated by * ($p < 0.05$) and # ($p < 0.005$). (E) Expression changes of VDR, CYP27B1, CYP24A1, RUNX-2, and OCN in proliferation, post-proliferation and differentiation stages.

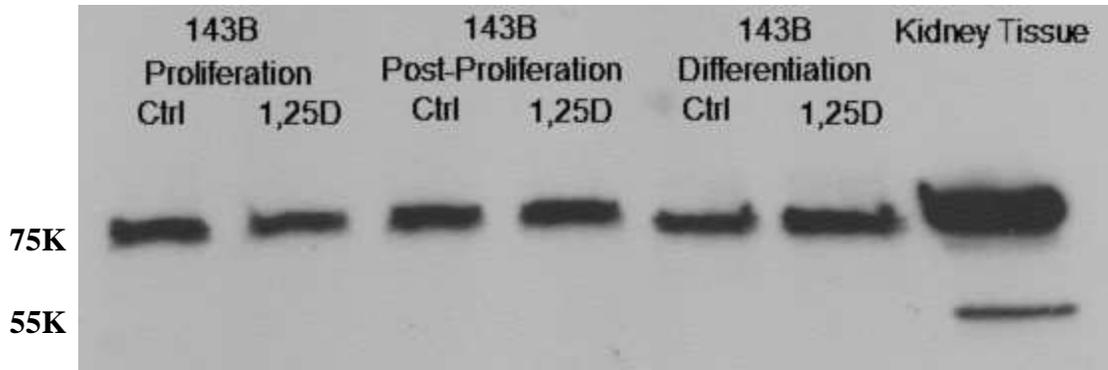
Protein Expression of Vitamin D Target Genes in Osteosarcoma 143B

Vitamin D target genes (VDR, CYP27B1, CYP24A1, RUNX-2, and OCN) are translated to protein at a detectable amount in 143B OS cells according to the results of western blot. The expected molecular weight of VDR is 55 kDa, of 1α -OHase is 56 kDa, of 24-OHase is 59 kDa, of RUNX-2 is 57 kDa, and of OCN is 55 kDa. All the proteins were detected at 75 kDa which are higher than their expected molecular weights. Presence of higher molecular weight forms of the above proteins in 143B cell lines might be due to post-translational modifications. Breast cancer cell line MCF-7 (Lane 1, Fig. 7A) cell lysate, and kidney (Lane 7, Fig. 7B, 7C,7E) tissue lysate were used as standards for VDR, 1α -OHase, and 24-OHase expression respectively. Expression of 1α -OHase in all the experiment samples was detected as 75 kDa band. In addition to the 75 kDa band, a 55 kDa band was also detected in kidney tissue lysate.

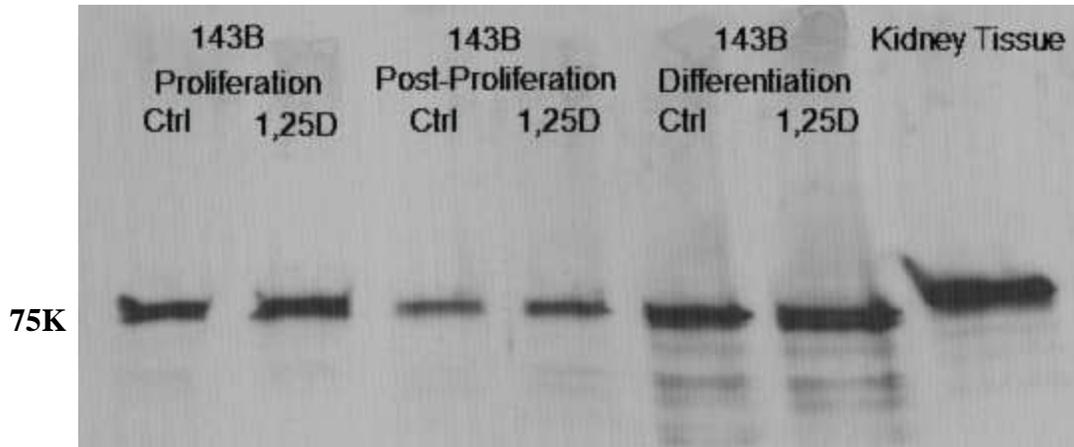
A. VDR



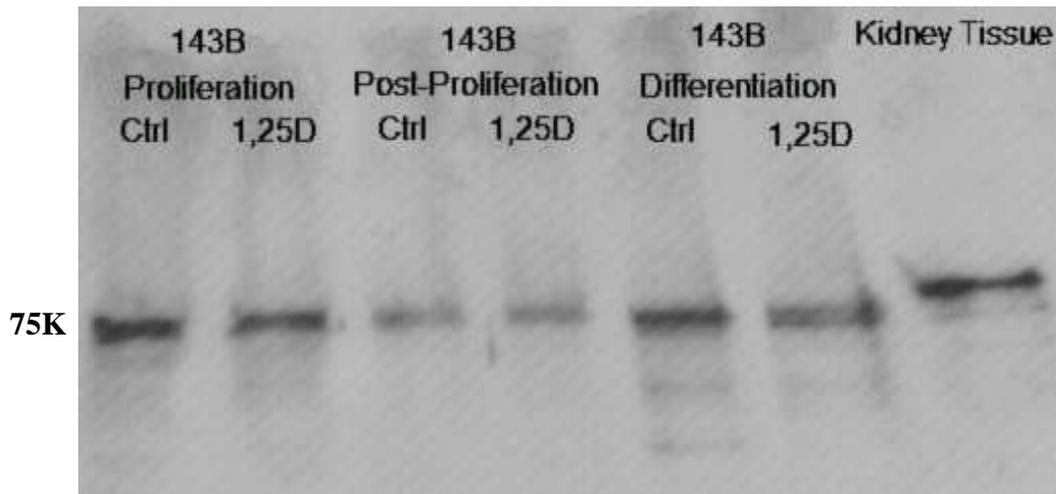
B. 1α -OHase



C. 24-OHase



D. RUNX-2



E. OCN

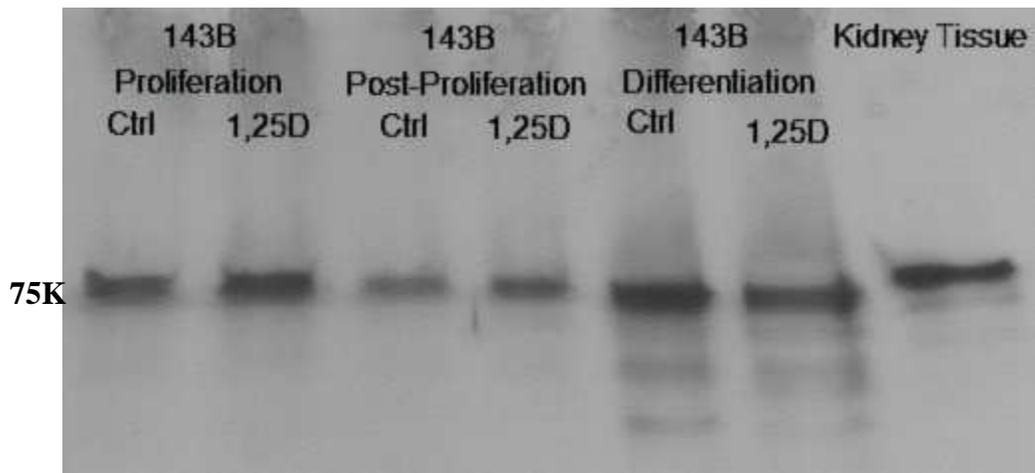


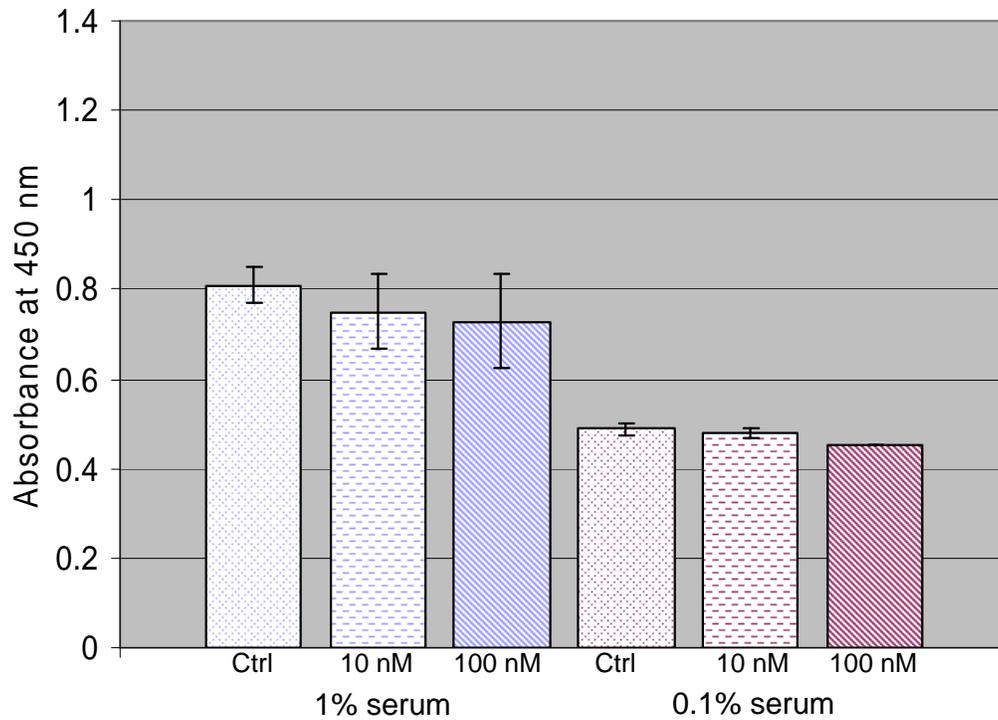
Figure 7. Effect of calcitriol on vitamin D target genes protein expressions. 143B was cultured in serum-reduced medium (supplemented with mito+ serum extender), with or without the addition of calcitriol (100 nM) for 3 days, 9 days, and 21 days, and processed for western blot analysis, for (A) VDR, (B) CYP27B1, (C) CYP24A1, (D) RUNX-2 and (E) OCN protein expression, as described.

Serum Masking Effects on Anti-Proliferation Activity of Calcitriol

In the cell proliferation MTS assay, cells were grown in serum-reduced

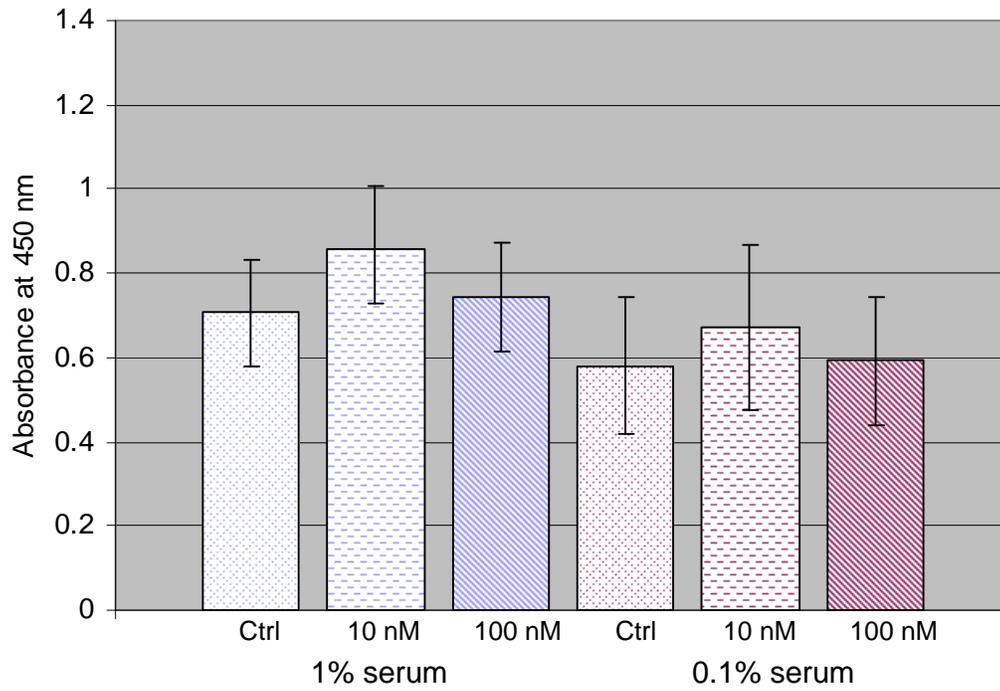
(supplemented with mito+ serum extender) medium for 4 days. Calcitriol has no significant effect on cell proliferation in all three osteosarcoma cell lines, SaOS-2, 143B, and U2OS, even at the higher concentration of 100 nM.

A.



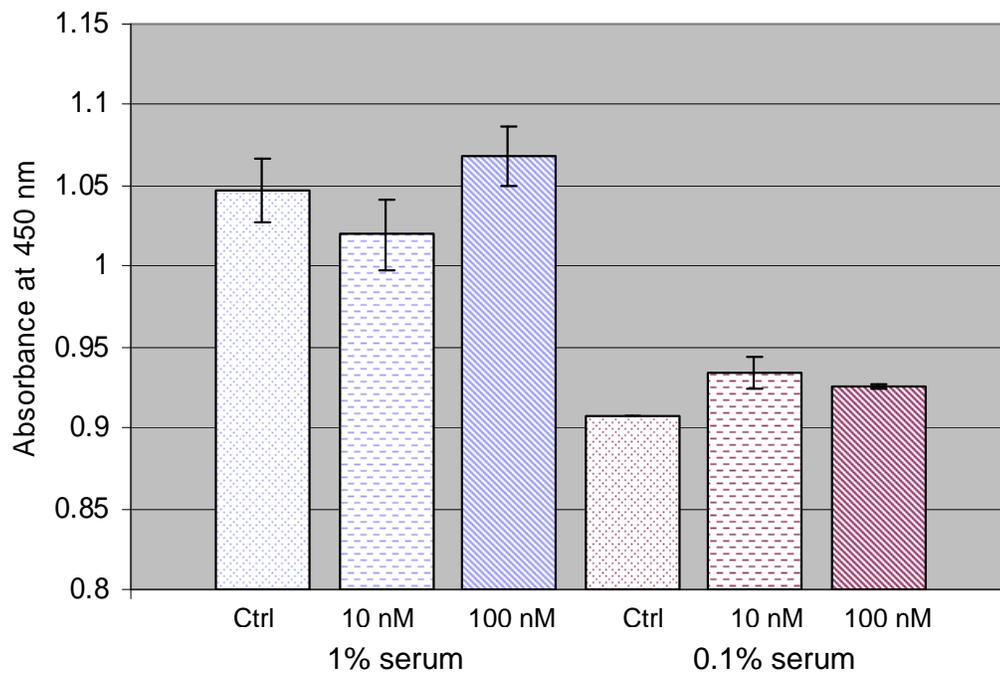
Serum concentration and effect of 1,25D on proliferation of SaOS-2 cells

B.



Serum concentration and effect of 1,25D on proliferation of 143B cells

C.



Serum concentration and effect of 1,25D on proliferation of 143B cells

Figure 8. MTS cell proliferation assay. Cells were cultured in serum-reduced medium (1% or 0.1 % FBS), with or without the addition of calcitriol at two concentration (10 nM and 100 nM) for 4 days, and processed for MTS assay.

Chapter 5

Discussion

In this study, we confirmed osteosarcoma cell line, 143B, contained the vitamin D regulatory system (VDR, CYP27B1, CYP24A1) and vitamin D responsive target genes and their encoded proteins (RUNX-2 and OCN) by real-time qPCR and western blotting studies. To our best knowledge, this is the first report of expression of vitamin D target genes (VDR, CYP27B1, CYP24A1, RUNX-2 and OCN) in the osteosarcoma 143B cell line.

The mRNAs of VDR, CYP27B1, and CYP24A1 are expressed by 143B cells in all three stages (Fig. 6A, 6B, 6C). No significant differences existed between control and calcitriol-treated groups in three stages. No significant effect was seen in a time-dependent manner except CYP27B1. CYP27B1 mRNA was significantly increased with time. Differences were significant between proliferation versus post-proliferation, proliferation versus differentiation groups, but not between post-proliferation versus differentiation when comparing control to control and calcitriol-treated to calcitriol-treated samples.

In proliferative and post-proliferative stages, there is a trend of increased expression of VDR in calcitriol-treated samples compared to controls. It is not significant. We can not see any trend of expression changes of CYP27B1 and CYP24A1 in all three stages.

Proteins of VDR, CYP27B1, and CYP24A1 were expressed in all three stages in 143B OS cell (Fig. 7A, 7B, 7C). It was consistent with the results of real-time qPCR. MCF-7 and kidney tissue lysate were used as a positive control for the proteins of VDR, 1 α -OHase, 24-OHase, RUNX-2, and OCN respectively. MCF-7 had a band of VDR at 55 kDa which was the same as the expected molecular weight. However, in 143B cells, molecular weight of VDR was about 75 kDa. In the 143B cell and kidney tissue lysate, the proteins of 1 α -OHase, 24-OHase, RUNX-2, and OCN were all at the 75 kDa band position. They were all higher than the expected molecular weights. The presence of the higher molecular weight forms might due to various post-translational modifications of protein occurring in different cancer cell types. In the human kidney and renal clear cell cancer (68), VDR is found at 60 kDa. In human sebocytes (69), VDR is demonstrated as a band at 53 kDa.

Protein amounts of VDR, 24-OHase, RUNX-2, and OCN in differentiation samples were higher than samples of the other two stages. Real-time qPCR results also indicated the greatest amount of mRNA expressions of these genes in differentiation. In post-proliferative stage, the protein expressions of VDR, 24-OHase, RUNX-2, and OCN were lower than those during proliferation stage. According to realtime qPCR results, VDR and CYP24A1 mRNA expressions were not significantly different between proliferation and post-proliferation. The potential reason for it might be: 1) the lower translation level of VDR and

CYP24A1 in post-proliferation, 2) the less amount of protein loading to the gel. Based on the western blotting result of 1-alpha-OHase, we cannot see significant difference of protein in three stages. That meant the protein amounts of each sample were the same. But it is still interesting for future studies.

RUNX-2 and OCN mRNA expressions were lower than the other vitamin D target genes in differentiation stage in 143B cells. It was possible that RUNX-2 and OCN mRNA were relatively unstable or they get rapidly translated to proteins (65). No significant differences existed between control and calcitriol-treated groups. According to western blotting results, RUNX-2 and OCN were expressed from 3 days with a detectable amount. Previous studies reported OCN expression in OS cell lines and osteoblast was highly variable. Ryhanen et al. (70) observed that OCN expression in MG-63 OS cells peaks within 24h of treatment with vitamin D analogue CB1093. Dass et al. (71) found no OCN expression in SaOS-2 and 143B cells in cultures, but could detect in tumor lesions induced by transplanted OS cells in immunodeficient mice. According to previous study in Dr. Garimella's lab, expression of osteogenic markers like alkaline phosphatase enzyme activity was seen by day 15 treated by calcitriol. More studies are needed to specify the results.

Our study indicates that calcitriol has no significant effects on gene expressions of vitamin D target genes (VDR, CYP27B1, CYP24A1, RUNX-2 and OCN) in all three cell growth stages in 143B cell line. A number of studies reported

the feedback regulation mechanism of vitamin D. In this study, CYP27B1 and CYP24A1 mRNA levels did not change between the control and calcitriol-treated groups. This result contradicted the general explanation of regulatory system of vitamin D. The inconsistent changes of CYP27B1 mRNA were also reported in primary human osteoblast NHBC cells (62) and kidney (63). According to the real time qPCR results in this study, CYP24A1 mRNA expressions were not consistent in 6 sets of samples (Data not shown). A huge error bar existed among them. Significant changes between control and calcitriol-treated groups existed in three groups, but not in the other three groups. More studies and samples are needed to specify the expression levels of CYP24A1 mRNA. In other cancers, CYP24A1 is regarded as a highly responsive vitamin D target gene. Another point that can be considered for future experiments is the timing of expression of vitamin D target genes.

Cell Proliferation MTS Assay

In this MTS assay, cells were grown in serum-reduced medium for 96 h. Calcitriol had no significant effect on cell proliferation in all three OS cell lines, SaOS-2, 143B and U2OS. In prostate cancer cells (66), calcitriol had biphasic effects on cell proliferation in the presence/absence of FBS. The results of MTS assay contradicted the anti-proliferative activity of calcitriol in breast, prostate, colon, skin, and brain cancer cells (11, 12). The difference was we used a

serum-reduced medium (1% serum and 0.1% serum) instead of serum-free medium. Even the medium contained a very low concentration of FBS, the factors present in FBS may interact with calcitriol and mask its antiproliferative effects. The reason for the lack of antiproliferative effect in our study might be due to an early termination (96 h). It was not long enough for calcitriol to exert its inhibitory effect on OS cells. Our results were consistent with recent studies by Peik et al. (72) clearly showing that calcitriol did not alter cell proliferation, but induced cell cycle changes in human mesenchymal stem cells.

Conclusion

Although many studies reported the anti-tumor activity of calcitriol in many cancer cell types (22-33), we did not find any significant effect of calcitriol on gene or protein expression of vitamin D target genes (VDR, CYP27B1, CYP24A1, RUNX-2, and OCN) in 143B OS cell.

Summary

Based on previous research on the anti-cancer effect of calcitriol in other tumor cells (22-33), we hypothesized calcitriol also has the effect in 143B OS cell and regulates the transcription and translation levels of vitamin D target genes (VDR, CYP27B1, CYP24A1, RUNX-2 and OCN). So we conducted this research to study the regulation exerted by calcitriol in vitamin D target genes by realtime

qPCR and western blotting. But no significant effect was found. Calcitriol did not exhibit the anti-cancer effect in 143B OS cell at 100 nM.

Limitation and Suggestion for Future Research

To our best knowledge, this is the first research to indicate expression of vitamin D target genes (VDR, CYP27B1, CYP24A1, RUNX-2 and OCN) in 143B OS cell. More studies are needed to validate the results and specify the underlying mechanisms in 143B OS cell.

This study is an *in vitro* study. It may overlook the complexity of *in vivo* tumor environment. The results need to be validated in animal models.

In this study, some real-time qPCR data had a huge error bar. But due to limit time, I can not include more samples to improve the results. It might be a sample error. Also, in the western blotting, as the protein amounts of each sample are not enough, thus we used stripping buffer to strip the antibody off and then probed with another antibody. In the Figure 7, we can see the protein bands of VDR, CYP24A1, RUNX-2, and OCN are almost the same. In order to confirm the former antibody was gone, after each stripping we used detection reagent to develop films for 5 min. Thus, it can verify that the bands are target proteins. For future studies, the western blotting should develop the membrane without stripping to specify the target proteins.

In all, this is a pilot study of study in expression level of genes in 143B OS

cell. Methods need to be optimized. Results should be demonstrated further before animal experiments.

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