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The Role of Vitamin D Receptors in Gastric epithelial Homeostasis

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THE ROLE OF VITAMIN D RECEPTORS IN GASTRIC EPITHELIAL HOMEOSTASIS

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Under the Supervision of Dr. Asma Al Menhali

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Declaration of Original Work

I, Ifrah Ismail Ali, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*The Role of Vitamin D Receptors in Gastric Epithelial Homeostasis*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Asma Al Menhali, in the College of Science at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Abstract

The gastric epithelium consists of different types of cells, which are involved in gastric homeostasis by balancing cell proliferation and differentiation. This process involves several signaling molecules such as growth factors, hormones and vitamins. Vitamin D_3 (VD_3) is engaged in several biological activities. It plays a role in cell differentiation, cell proliferation, immune response and also regulates calcium homeostasis. The biological activities of VD_3 are mediated by vitamin D receptor (VDR). Target tissues of VD_3 in the gastrointestinal tract were identified earlier in intestine, colon and gastric cancer tissues; however, the normal expression of VDR in stomach is poorly studied. So, the main objectives of this thesis are: 1) to investigate the normal expression, distribution and cellular localization of VDR in gastric epithelium and 2) to study possible role of VDR and VD_3 in maintaining gastric stem cells proliferation and differentiation by establishing and analyzing mouse model deficient in VD_3. Polymerase chain reaction (PCR) analysis showed that VDR as well as enzymes involved in VD_3 metabolism are expressed in the different region of normal mouse stomach. Co-immunostaining analysis showed specific expression of VDR in the acid secreting parietal cells and the different mucus secreting cells. The results suggested that parietal cells and mucous cells are targets for VD_3 signaling. To examine the role of VD_3 on gastric homeostasis, wild type mice were put on VD_3 deficient diet for 3 months. Using Real-Time Polymerase Chain Reaction (Real-Time PCR), stomachs of mice deficient of vitamin D showed significant decrease in expression of parietal cell specific genes (*HKα* and *HKβ*) and increase gastrin gene expression. Moreover, quantification for cells in the S-phase of the cell cycle showed significant increase in their number in vitamin D deficient mice compared to controls. Gene expression analysis of VDR signaling genes showed significant decrease in *PTHLH*, but not other target genes like *TRPV6* and *p21*. This work will add value to the field of stomach biology by providing better understanding of how VD_3 and VDR are involved in maintaining gastric epithelial homeostasis and how that is related to some stomach conditions such as low gastric acidity and gastric cancer.

Keywords: Vitamin D_3, VDR, Gastric epithelium, Stem cell, Proliferation, Differentiation.

Title and Abstract (in Arabic)

دور مستقبلات فيتامين د في توازن الخلايا المعدية

الملخص

تتكون بطانة المعدة من عدة أنواع من الخلايا التي تساهم في الحفاظ على توازنها عن طريق الموازنة بين تزايد أعداد هذه الخلايا و بين تمايزها الى خلايا أخرى متخصصة. تساهم في هذه العملية العديد من الجزيئات: مثل عوامل النمو و الهرمونات و الفيتامينات. يلعب فيتامين د (VD_3) دوراً مهماً في العديد من الانشطة البيولوجية، حيث يلعب دورًا في تمايز الخلايا وتتزايدها والاستجابة المناعية وينظم أيضًا توازن الكالسيوم في الجسم. يتوسط هذه النشاطات البيولوجية لفيتامين د مستقبلاتها الخاصة والتي تعرف بمستقبلات فيتامين د (VDR) . من خلال الابحاث السابقة تم تحديد الأنسجة المستهدفة من فيتامين د في الجهاز الهضمي والتي تتمثل بالأمعاء و القولون والانسجة السرطانية في المعدة، ولكن دور هذه المستقبلات في المعدة لم يتم دراسته بشكل كافي. تتمحور الأهداف الرئيسة لهذه الأطروحة حول: 1) التحقق من تواجد مستقبلات فيتامين د في أنسجة وخلايا المعدة وأيضا 2) دراسة دور مستقبلات فيتامين د في الحفاظ على توازن الخلايا الجذعية للمعدة، من حيث تزايد أعدادها وتمايزها عن طريق تطبيق بعض التقنيات مثل صبغ أنسجة المعدة بأنواع خاصة من الأجسام المضادة (Immunohistochemistry) و دراسة معدل تغير بعض الجينات (Real-Time PCR). من خلال استخدام نموذج لفئران تعاني من نقص فيتامين د أظهرت نتائجنا أن مستقبلات فيتامين د بالإضافة إلى الإنزيمات المشاركة في عملية تكوين فيتامين د تتواجد في أجزاء المختلفه من المعدة. كما أظهرنا انخفاض كبير في تواجد الحمض النووي الرايبوزي من النوع (mRNA) للجينات ($HK\beta$) و ($HK\alpha$) و ارتفاع الحمض النووي الرايبوزي للجين (*gastrin*) في الفئران التي تعاني من نقص فيتامين د. وعلاوة على ذلك ، أظهرت النتائج التي توصلنا إليها زيادة كبيرة في عدد الخلايا الجذعية و أيضا تراجع النسبيي لبعض الجينات المستهدفة من فيتامين د مثل (*TRPV6*، *p21*، *PTHLH*) فقط في الفئران التي تعاني من نقص في فيتامين د. سيضيف هذا العمل قيمة إلى حقل الأبحاث من خلال توضيح كيفية مشاركة فيتامين د و مستقبلات فيتامين د في الحفاظ على توازن الخلايا الجذعية في المعدة وكيف يرتبط ذلك ببعض الحالات الصحية مثل انخفاض حموضة المعدة وسرطان المعدة.

مفاهيم البحث الرئيسية: فيتامين د، مستقبلات فيتامين د، خلايا بطانة المعدة، الخلايا الجذعية، تزايد الخلايا، تمايز الخلايا.

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Dedication

To my mentor Dr. Asma Al Menhali for being a source of knowledge and inspiration, to my parents for their support and continuous prayers, to all my beloved family

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List of Abbreviations

AF-1	Ligand-independent transactivation Function domain
AF-2	Ligand-dependent transactivation Function domain
AP	Alternative Pocket
BrdU	5-bromo-2'-deoxyuridine
CAMP	Cathelicidin Antimicrobial Peptide
CYPs	Cytochrome P450 enzymes
CYP2R1	25-hydroxylase
CYP27A1	Sterol 27-hydroxylase
CYP27B1	1α-hydroxylase
CYP24A1	24-hydroxylase
DAB	3'-Diaminobenzidine
DBD	DNA Binding Domain
DBP	Vitamin D Binding Protein
DNA	Deoxyribonucleic Acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GI Tract	Gastrointestinal tract
GP	Genetic Pocket
HK	H^+K^+ATPase
HKα	H^+K^+ATPase sub-unit α
HKβ	H^+K^+ATPase sub-unitβ
IF	Intrinsic Factor
IHC	Immunohistochemistry
LBD	Ligand Binding Domain

LCA	Lithocolic Acid
LC–MS/MS	Liquid chromatography tandem-mass spectrometry
mGEP	Mouse Gastric Epithelial Progenitor
Muc5AC	Mucin 5, subtypes A & C
Muc6	Mucin 6
p21	Cyclin-Dependent Kinases Inhibitor
PCR	Polymerase Chain Reaction
PTH	Parathyroid Hormone
PTHLH	Parathyroid Hormone Like Hormone
RANKL	Receptor Activator of Nuclear factor Kappa-B Ligand
Real-TimePCR	Real-Time-Polymerase Chain Reaction
RNA	Ribonucleic Acid Reverse
RT-PCR	Reverse Transcription- Polymerase Chain Reaction
RXR	Retinoid X Receptor
SDD	Standard Diet/Dark group of mice
SDL	Standard Diet/Light group of mice
TRPV6	Transient Receptor Potential Vanilloid member 6
UV-B	Ultra Violet radiation - B
VD_2	All forms of vitamin D_2
VD_3	All forms of vitamin D_3
VDD	Vitamin D_3 deficient Diet/Dark group of mice
VDR	Vitamin D Receptor
VDREs	Vitamin D Response Elements
$1\alpha,25(OH)_2D_3$	1α,25-dihydroxyvitamin D_3

$25(OH)D_3$	25-hydroxyvitamin D_3
CYP7A1	Cholesterol 7a-hydroxylase

Chapter 1: Introduction

1.1 Overview

Vitamins are organic compounds with several metabolic functions, they are distinct from minerals, amino acids and fatty acids [1]. Vitamins are nutritional factors, naturally present in the diet, required in small quantities and essential for the body to perform its ordinary physiological functions such as growth, reproduction and maintenance. It is remarkable to realize that not all vitamins are found in diet, some of them need further activation in our body to be converted into their functional form. There are various types of vitamins such as vitamin A, vitamin B1, vitamin B2, vitamin B6, vitamin B12, vitamin C, vitamin D, vitamin E, vitamin K, Niacin, Biotin, Folic acid and Pantothenic acid [2].

Some of these vitamins such as biotin and vitamin B6 act as coenzymes for carboxylations, fatty acid metabolism or amino acid metabolism [2]. Moreover, vitamin A is essential for the growth and eye vision [3].

The classical effect of vitamin D is very much connected to bone structure and regulation of calcium homeostasis [4]. Nevertheless, vitamin D is also involved in several cellular processes, such as cell differentiation [5] and proliferation [6]. Moreover, vitamin D contributes significantly to many physiological functions like pancreatic secretion of insulin [7], in cardiovascular system [8] as well as immune response [9].

This introductory chapter is shedding light on vitamin D_3 and vitamin D receptor. It begins with brief historical overview of vitamin D, followed by description of its structure, metabolism process as well as how its function is meditated by vitamin D receptor. The role of the ligand and its receptor in health and disease will be

illustrated as well. Furthermore, throughout the chapter the expression and the function of vitamin D receptor in gastrointestinal tract and other tissues will be demonstrated.

1.2 Vitamin D

The early discovery of vitamin D started with a series of experiments aimed to study rickets disease; which is defective calcification of bones, in different models. A classical experiment conducted by Edward Mellanby involving two months old puppies which were provided with specific diet known to produce rickets. Another group of puppies was fed the same diet in addition to other kinds of food such as meat, malt extract, butter, yeast extract and cod liver oil. The puppies were kept indoor inadvertently. The observation was that the group of puppies fed diet with cod liver oil or butter didn't develop rickets at all Rickets was diagnosed by using X-ray. The conclusion of this work was that the presence of certain fat-soluble accessory factors such as cod liver oil or butter in the diet can prevent rickets disease [10].

McCollum also discovered earlier that some fats contain dietary substances which are involved in the growth process [3]. In 1919, he found that cod liver oil contains substance which could prevent rickets and named it vitamin D [11].

Other than the supplementary factors in the diet, Harriette Chick and her colleagues [12] as well as other researchers [13] demonstrated the effect of sunlight in curing rickets in children. So the dominated idea at that time was that some dietary factors such as cod liver oil and sunlight can attribute in preventing and curing rickets. Furthermore, to investigate whether the fatty dietary factors from plants represented by phytosterol can have anti-rachitic effect just like animal fat (i.e. cod liver oil) as noticed earlier, Alfred Hess and his team prepared rickets producing diet along with phytosterol extracted from cottonseeds. A group of rats was fed an UV-irradiated

extract of this diet, while another group was fed non-irradiated extract. The same experiment was repeated by using cholesterol extracted from brain tissue. They noticed that the group of rats which had irradiated phytosterol or cholesterol didn't produce rickets disease unlike the other group, concluding that the irradiation of the fatty substance is a critical step for the rickets prevention. Furthermore, they hypothesized that cholesterol which is present in our skin cells can be converted to the active form of cholesterol by UV irradiation, and then circulated in the body in order to prevent rickets [14]. Thereafter, it was discovered that the precursor molecule is not cholesterol, but in fact another impurity found within the cholesterol which acts as anti-rickets factor and was named as provitamin [15]. Moreover, the provitamin should be irradiated by UV light to be converted to vitamin D [16].

1.2.1 Vitamin D_2 and Vitamin D_3

Vitamin D is widely described as hormone since it is derived from pro-steroid. There are two different forms of vitamin D: Vitamin D_2 , Ergocalciferol [17] and Vitamin D_3 , Cholecalciferol [18]. Ergocalciferol is a plant based form of vitamin D, it is derived from the provitamin ergosterol which is converted into ergocalciferol by UV irradiation [17].

On the other hand, Cholecalciferol is produced in the epidermis of animals and humans by the photolysis of the sterol precursor; 7-dehydrocholesterol [18]. Ergocalciferol and Cholecalciferol have slight differences in their structure. The absence of double bond between C22 and C23 and absence of methyl group at C24 in Cholecalciferol lead to a distinct side chain for each of vitamin D forms (Figure 1.1). However, both can be converted to 25-hydroxyvitamin D [18], [19].

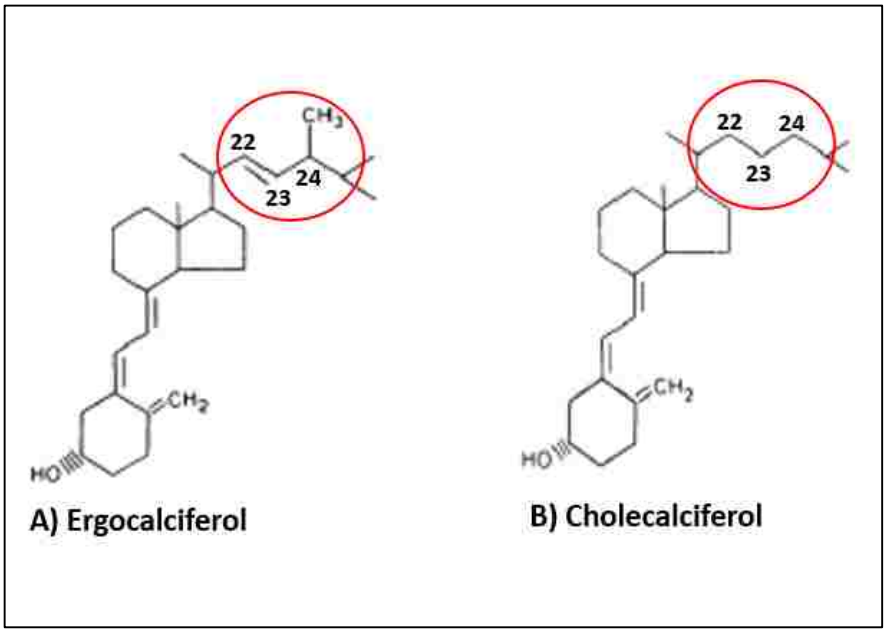

Figure 1.1: Molecular structure of the two forms of vitamin D.
(A) Ergocalciferol (B) Cholecalciferol. Ergocalciferol is characterized by having double bond between C22 and C23 and methyl group at C24 [18].

1.2.2 Vitamin D Metabolism

Ergocalciferol and Cholecalciferol can be obtained from dietary sources, however, the main source of Cholecalciferol is the skin. The provitamin molecule; 7-dehydrocholesterol is naturally present in animal cells, specifically in the skin, and works as precursor for Cholecalciferol. After the exposure to sunlight, ultra-violet radiation B (UV-B) converts 7-dehydrocholesterol to previtamin that has unstable structure and consequently isomerizes to form Cholecalciferol in the plasma membrane of the skin cells. As Cholecalciferol leaves the plasma membrane to the extracellular fluid space, it binds to Vitamin D Binding Protein (DBP) in the blood stream for translocation [20]. Illustration of vitamin D activation is demonstrated in (Figure 1.2).

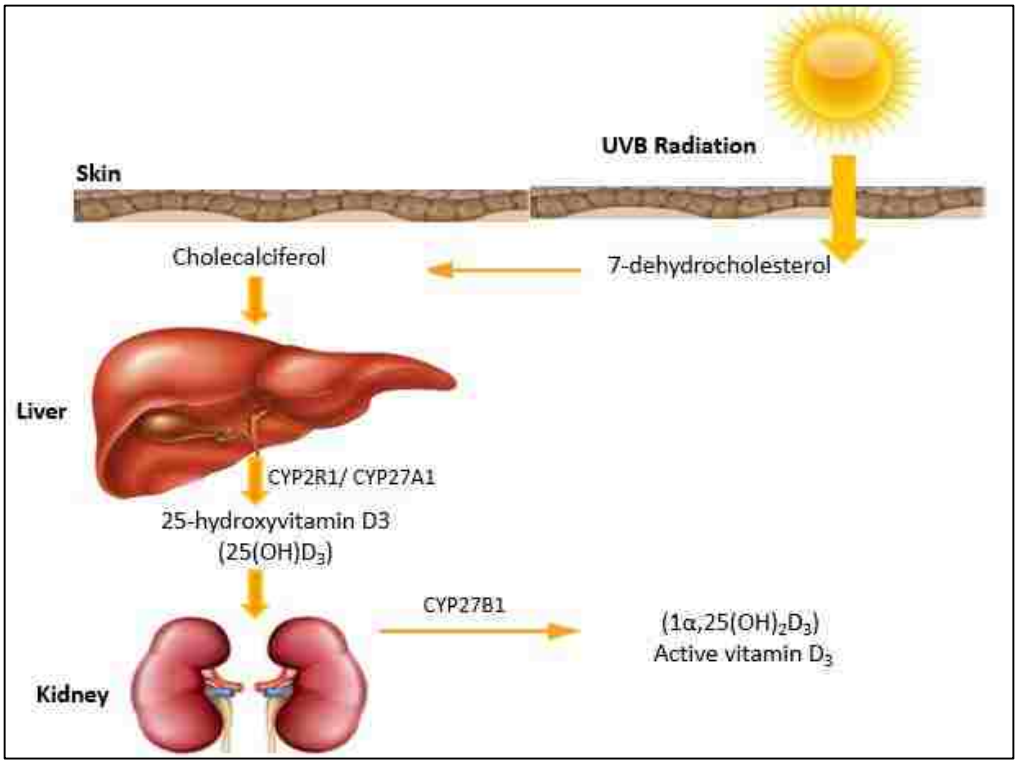

Figure 1.2: Vitamin D_3 synthesis pathway.
UVB radiation converts 7-dehydrocholesterol to Cholecalciferol through photochemical reaction. CYP2R1 and CYP27A1 enzymes synthesize 25-hydroxyvitamin D_3 ($25(OH)D_3$) from Cholecalciferol in liver. 25-hydroxyvitamin D_3 ($25(OH)D_3$) is translocated to kidney to be further synthesized to the active form of vitamin D_3; 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$) by the enzyme CYP27B1.

Thereafter, Cholecalciferol is translocated to the liver where the enzymes 25-hydroxylase (CYP2R1) and sterol 27-hydroxylase (CYP27A1) convert Cholecalciferol to 25-hydroxyvitamin D_3 ($25(OH)D_3$). As $25(OH)D_3$ is synthesized, it is transported mainly to the kidney via DBP where it is further hydroxylated to produce the biologically active form of VD_3; 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$) by the enzyme 1α-hydroxylase (CYP27B1); an enzyme mainly expressed in the kidney. Then $1\alpha,25(OH)_2D_3$ travels to vitamin D target tissues such as bone and intestine to perform its biological functions which are mediated by Vitamin D Receptor (VDR) (Figure 1.2) [21]. $1\alpha,25(OH)_2D_3$ is lipophilic steroid hormone with long and flexible structure [22].

As demonstrated earlier, there are distinct key proteins involved in vitamin D metabolism, these proteins include some cytochrome P450 enzymes (CYPs) as well as DBP. Cytochrome P450 2R1 enzyme (CYP2R1), also known as vitamin D 25-hydroxylase is encoded by the gene CYP2R1, this microsomal hepatic enzyme has vitamin D 25-hydroxylase activity and it is the major contributor to $25(OH)D_3$ synthesis [23], [24]. Zhu *et al* showed that more than 50% decline in serum $25(OH)D_3$ level and steady level of serum $1\alpha,25(OH)_2D_3$ in *CYP2R1*$^{-/-}$ mice comparing to wild type. These knockout mice had no other serious health problems [24]. While in human, mutation in *CYP2R1* gene developed symptoms of vitamin D-dependent rickets in children as illustrated in a clinical study [25].

Cytochrome P450 27A1 enzyme (CYP27A1) which commonly known as sterol 27-hydroxylase is mitochondrial hepatic enzyme primarily responsible for bile acid formation [26]. Additionally, this enzyme also has the ability to stimulate the conversion of Cholecalciferol to $25(OH)D_3$ by hydroxylation [27]. In a previous study, *CYP27A1*$^{-/-}$ mice experienced disruption in bile acid formation process and normal vitamin D metabolites level [28]. Suggesting that CYP27A1 enzyme might be a minor contributor in $25(OH)D_3$ formation.

Concluding, CYP2R1 and CYP27A1 which has different hepatic intracellular localization are engaged in the enzymatic hydroxylation of Cholecalciferol to produce $25(OH)D_3$ in liver.

1α-hydroxylase (CYP27B1) is another enzyme from Cytochrome P450 super-family, expressed in the proximal tubules of kidney, interestingly CYP27B1 is the sole enzyme responsible for synthesizing $1\alpha,25(OH)_2D_3$ [21]. On the other hand, the activity of CYP27B1 can be suppressed by serum $1\alpha,25(OH)_2D_3$ [29] as well as high calcium levels in blood [30].

Interestingly, 1α,25$(OH)_2D_3$ and 25$(OH)D_3$ degradation is initiated by Cytochrome P450 24 A1 enzyme (CYP24A1); 24-hydroxylase when necessary [31]. This enzyme is responsible for the catabolism of vitamin D, as it is capable of C24 or C23 site hydroxylation. One of the final products of 1α,25$(OH)_2D_3$ catabolism is calcitroic acid; as a result of C24 hydroxylation [32].

Several studies showed that the expression of these enzymes is not specific for the liver and kidney. In vitro studies illustrated that *CYP27B1* is expressed in HaCaT keratinocytes [33], macrophages [34] as well as bone [35]. While *CYP2R1*, *CYP24A1* and *CYP27B1* are expressed in placenta [36].

Another important protein in vitamin D metabolism and action is DBP. DBP is from Group specific component proteins, this plasma protein has the ability to bind to vitamin D and its metabolites serving in transporting them between tissues through blood stream [37].

Table 1.1: Molecules involved in vitamin D_3 synthesis

Molecule	Location of synthesizing	Activated/ synthesized by	Precursor molecule	Reference
7-dehydrocholesterol	Skin	Sun (UVB radiation)	-	[20]
Cholecalciferol	Skin	Sun (UVB radiation)	7-dehydrocholesterol	[20], [18]
25-hydroxyvitamin D_3 ($25(OH)D_3$)	Liver	CYP2R1 and CYP27A1 enzymes	Cholecalciferol	[21], [23], [27]
1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$)	Kidney	CYP27B1 enzyme	25-hydroxyvitamin D_3	[21], [22]

1.3 Vitamin D Receptor

The biological activities and functions of $1\alpha,25(OH)_2D_3$ are mediated by VDR. VDR is a member of nuclear receptors family; member 1 from nuclear receptor subfamily 1, group I [38]. The function of VDR is highly correlated with its structure and molecular nature. VDR has two conserved binding domains, Ligand Binding Domain (LBD) and DNA Binding Domain (DBD) [39].

LBD represents the site where $1\alpha,25(OH)_2D_3$ or its analogs can bind, heterodimerization with Retinoid X Receptor (RXR) occur and VDR-mediated transcription activation take place throughout ligand-dependent transactivation Function domain (AF-2). Whereas DBD is involved in the process of target genes activation through the direct binding to the DNA via two zinc fingers [39], [40]. In addition to these conserved domains, there are ligand-independent transactivation function domain (AF-1) and Hinge domain which allow flexible rotation for the receptor [40] (Figure 1.3).

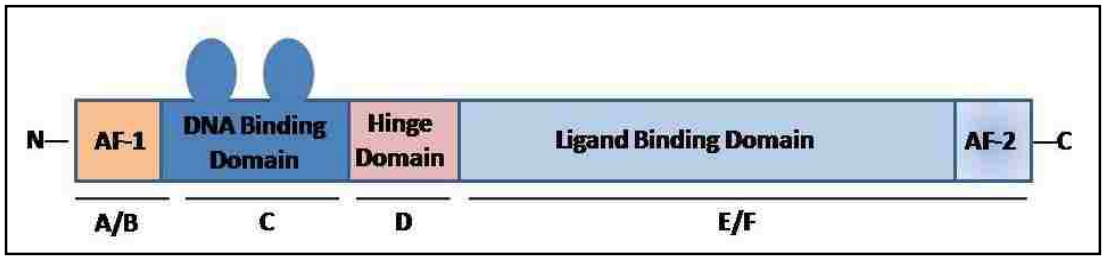

Figure 1.3: Graphic representation of VDR structure.
AF-1 is toward N terminus site. DBD contains two zinc fingers which allow proper binding to target genes. Hinge Domain provide rotational flexibility of VDR. ligands and RXR bind to LBD, while coactivators interaction take place at AF-2 site.

Interestingly, VDR-LBD has two overlapping pockets; Genetic Pocket (GP) (which is responsible of genetic responses when VDR acts as transcription factor), and Alternative Pocket (AP) (which is involved in rapid responses [41] such as chloride

channel regulation [42] and rapid intestinal calcium absorption) [43]. The activation of VDR results from binding of $1\alpha,25(OH)_2D_3$ to either pockets [44].

1.3.1 Mechanism of Action

Throughout this thesis, the active form of VD_3 will be stated as $1\alpha,25(OH)_2D_3$. VDR serves as ligand-dependent transcription factor when $1\alpha,25(OH)_2D_3$ binds to the GP, leading to an interaction between the complex $1\alpha,25(OH)_2D_3$ and VDR with another receptor know as Retinoid X Receptor (RXR). That interaction drives to $1\alpha,25(OH)_2D_3$-VDR-RXR heterodimer formation. The heterodimerized complex travels to the nucleus where it recognizes and binds to Vitamin D Response Elements (VDREs) located in the promoter of vitamin D direct target genes. As a result, the activated VDR induces coactivators or corepressors to regulate the transcription of the target genes directly (Figure 1.4). The transcription of these genes leads to the production of proteins responsible for mediating the genetic functions of VDR [44],[45]. VDREs is made up of two half-sites of similar, but not identical six nucleotides separated by three nucleotides spacer [46].

VDR is also considered plasma membrane receptor, since it is associated with the plasma membrane caveolae initiating rapid response (non-genetic mechanism). As soon as the ligand binds to the AP, a rapid signal transduction pathways will be activated such as rapid absorption of Ca^{2+} [44].

The ability of one ligand to induce two different responses when binding to a single receptor is centered around the capability of $1\alpha,25(OH)_2D_3$ to undergo conformational changes due to its flexible structure [47].

1.3.2 Vitamin D Target Genes

The biological responses toward VD_3 are mediated by VDR. $1\alpha,25(OH)_2D_3$ regulates transcription of several genes through VDRE on the promoter of these target genes. Example of these target genes is Transient Receptor Potential Vanilloid member 6 (*TRPV6*), which is Ca^{2+} channel that plays a role in Ca^{2+} uptake in GI tract [48]. Chow *et al.* illustrated that capsaicin can induce apoptosis in gastric cancer cells through *TRPV6* mediated mechanism [49]. Parathyroid Hormone Like Hormone (*PTHLH*) is another gene which is transcriptionally regulated by $1\alpha,25(OH)_2D_3$ [50]. *PTHLH* is involved in chondrocyte cells differentiation and proliferation and bone resorption [51].

Moreover, Cyclin-Dependent Kinases Inhibitor (*p21*); a tumor suppressor that cause cell cycle arrest is regulated by $1,25(OH)_2D_3$ [52]. $1,25(OH)_2D_3$ has antiproliferative activities by modulating the expression of *p21* via VDREs and further having cell cycle regulatory effect [53].

Cathelicidin Antimicrobial Peptide (*CAMP*) gene is another vitamin D target gene with VDREs [54]. These small peptides are stored in granules produced by neutrophils in the skin and other tissues. They play an essential role in immune response against viral and bacterial infection [55].

Moreover, cytochrome P450 3A4 (*CYP3A4*) is a gene expressed in liver as well as intestine, directly regulated by vitamin D through VDREs [56]. *CYP3A4* is mainly involved in breaking down the toxic agent; secondary bile acid Lithocolic Acid (LCA). This detoxification role of *CYP3A4* is modulated by pregnane X receptor and nuclear xenobiotic [57]. Furthermore, $1,25(OH)_2D_3$ is a potent and direct inducer of *RANKL* gene expression in osteoblastic cells [58]. The heterodimer VDR and act one *RANKL*

promoter initiated osteoclast differentiation supporting its survival, and consequently enhancing bone resorption [58].

Table 1.2: Some vitamin D_3 target genes

Target Gene		Function	Reference
PTHLH	Parathyroid Hormone Like Hormone	Involved in stimulating bone resorption. Induce myorelaxant effects in smooth muscles of different organs.	[59]
TRPV6	Transient Receptor Potential Vanilloid member 6	Highly Ca^{2+} selective channel protein that plays major role in Ca^{2+} uptake in kidney and gastrointestinal tract.	[48]
p21	Cyclin-Dependent Kinases Inhibitor	Tumor suppressor, that cause cell cycle arrest, by inhibiting the cells to enter S phase.	[60]
CAMP	Cathelicidin Antimicrobial Peptide	Peptide agent protective against microbial infections, produced by neutrophils in skin, aquamouse epithelial on mouth, vagina and other tissues.	[55]
CYP3A4	Cytochrome P450 3A4	Detoxification, by breaking down the hepatotoxic acid; Lithocholic Acid.	[56]
RANKL	Receptor Activator of Nuclear factor Kappa-B Ligand	Essential for bone resorption.	[58]

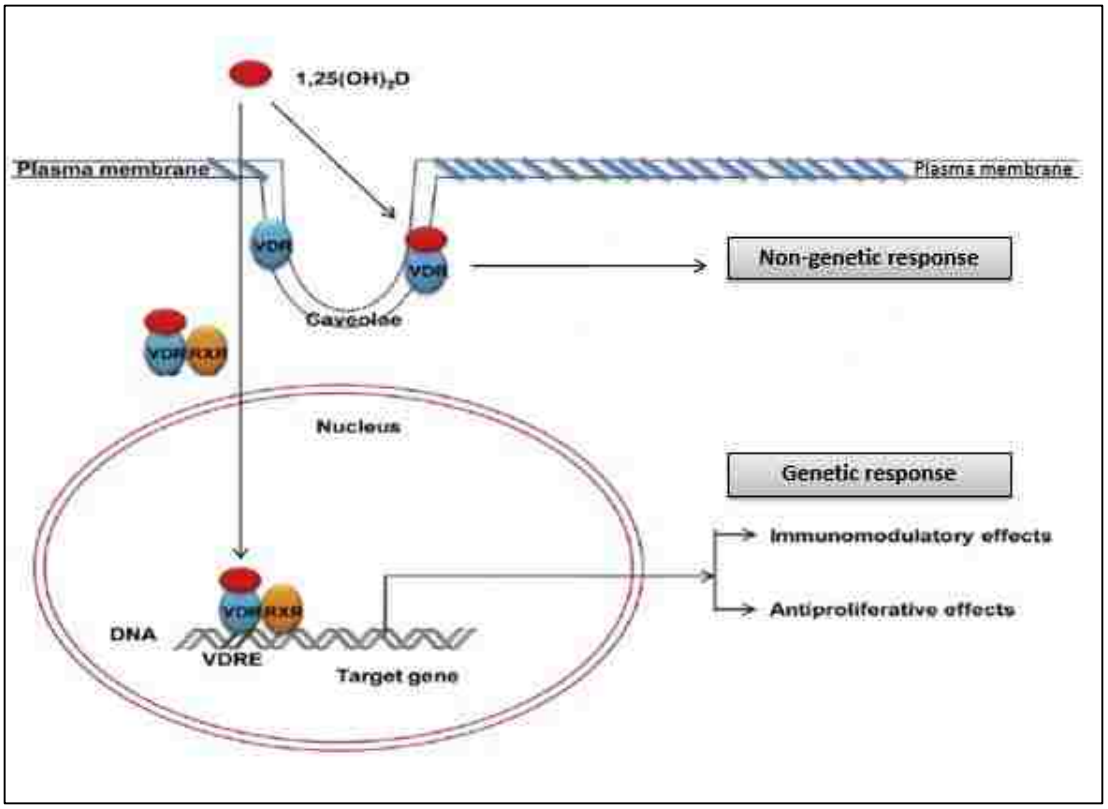

Figure 1.4: VDR genetic and non-genetic mechanisms.
For the genetic action, the ligand $1\alpha,25(OH)_2D_3$ binds to VDR, then the complex interact with RXR leading to form heterodimer. VD_3-VDR-RXR heterodimer travel to the nucleus where it recognizes VDREs of the target gene and binds there inducing coactivators or corepressors to regulate the transcription. For the non-genetic action, $1\alpha,25(OH)_2D_3$ binds to VDR which is associated with the plasma membrane caveolae inducing rapidly response (Modified from [61]).

1.4 Vitamin D and VDR in Health and Disease

It is well known that vitamin D plays a major role in maintaining calcium homeostasis and bone health. In addition, researchers were very much interested to study the non-skeletal actions of vitamin D, showing evidence that vitamin D status and VDR are associated with multiple biological responses such as cell proliferation [6], differentiation [62]. Vitamin D is also important for in the nervous system, immune system [62] and cardiovascular system [63].

1.4.1 Calcium Homeostasis

The classical action of vitamin D is to maintain calcium (Ca^{2+}) and phosphorus (P) serum levels and bone mineralization. Once serum Ca^{2+} level decreases, Parathyroid Hormone (PTH) is released acting on kidney to decrease loss of Ca^{2+} in urine. Moreover, PTH acts on stimulating bones to mobilize Ca^{2+} from it and send it directly to the blood. In addition to that, $1\alpha,25(OH)_2D_3$ can directly bind to VDR on intestine leading to Ca^{2+} and P uptake. Finally these actions return Ca^{2+} to its normal level [4].

1.4.2 Cell Differentiation

Far beyond the classical action, vitamin D can induce cell differentiation. Wang *et al* showed that $1\alpha,25(OH)_2D_3$ has the ability to induce differentiation of monocyte of human myeloid cells in HL60 cells. $1\alpha,25(OH)_2D_3$ increased the expression of Kinase Suppressor Of Ras 1 (*KSR-1*) gene which has VDREs, stimulating monocytic differentiation [62].

1.4.3 Cancer

Studies with system models of lung cancer [64], prostate cancer [65], breast cancer [66] as well as hyperplasia of parathyroid gland [6] showed that $1\alpha,25(OH)_2D_3$ has anticancer effects by down-regulating the hyper-proliferative cells and promoting apoptosis. Additionally, patients with pancreatic ductal adenocarcinoma showed upregulation of *CYP24A1* mRNA in malignant tissues comparing to nonmalignant tissues of the pancreas. As a result, reducing the antiproliferative effect of $1\alpha,25(OH)_2D_3$ in malignant tissues [67].

1.4.4 Nervous System

Knowing that VDR is expressed in neurons [68], [69], [70], recent studies suggested that some neurodegenerative diseases such as Alzheimer, Parkinson and multiple sclerosis diseases are linked to Vitamin D deficiency [71]. These neurodegenerative diseases are characterized by increased Ca^{2+} signaling [71]; and since vitamin D controls the expression of Ca^{2+} regulating proteins [72], progress of these diseases might be expected when there is low vitamin D in the body.

1.4.5 Cardiovascular System

Some studies demonstrated that low vitamin D levels due to insufficient UV light intensity and dysregulation of the reactive oxygen species and Ca^{2+} signaling may contribute to the pathogenesis of some cardiovascular diseases such as hypertension, cardiac hypertrophy, congestive heart failure and atrial arrhythmias [63], [71].

1.4.6 Depression

In psychiatric conditions, vitamin D deficiency or insufficiency has been

studied in depression cases. Milaneschi *et al* Showed association between 25(OH)D serum level and depression in a study which involved group of individuals (1,102 with current depressive disorders, 790 with previous depressive disorders, 495 control) aged 18-65 years. The investigation showed lower 25(OH)D serum level among the individuals with current depressive disorders as compared with the other groups [73].

1.4.7 Hair Loss

Moreover, studies in animals and human reported that vitamin D is linked to hair loss. Xie *et al* demonstrated that lack of VDR affected hair growth by inhibiting keratinocyte differentiation; consequently, preventing keratin lament bundles formation in VDR knockout mouse. The phenotypes included expansion of hair follicle with dermal cysts formation causing complete alopecia with time [74].

Furthermore, a study done on 80 females age between 18 to 45 years old with hair loss disease; female pattern hair loss (FPHL) or chronic telogen effluvium (TE), showed that vitamin D_2 serum level was significantly lower in these females compared to control group [75].

1.4.8 VDR Polymorphysims

In human, alterations in DNA sequence of *VDR* gene can cause major defects in the gene activities. Point mutation in *VDR* gene has been linked to heredity vitamin D-resistant rickets which is famous with insensitivity to $1\alpha,25(OH)_2D_3$ by the target tissues [76].

Diabetes Mellitus which is an endocrine-chronic disease has been also linked to *VDR* polymorhysims. Early studies showed that in pancreas, insulin producing β cells are expressing *VDR* [77]. Interestingly, it was found that insulin secretion reduced

in *VDR* mutant mice [78] concluding that VDR plays a role in insulin secretion process.

Moreover, an epidemiological study demonstrated an association between low vitamin D level and decreased insulin sensitivity in healthy group with specific *VDR* polymorphysim [79]. Another study had linked type 1 diabetes mellitus and *Bsm*I-*VDR* polymorphysim among diabetic Taiwanese population [80].

Furthermore, it was described that *VDR* polymorphisms has been associated with secondary hyperparathyroidism in chronic renal failure, some cancers such as breast and colon as well as some autoimmune diseases [81].

1.5 VDR in Gastrointestinal Tract

Target tissues of vitamin D throughout the gastrointestinal tract (GI tract) were identified earlier in different animal models [82]. Animals injected with radiolabeled $1\alpha,25(OH)_2D_3$ followed by microscopic autoradiography examination showed many ligand binding sites in the small intestine, stomach and colon [82].

VDR expression was reported in several tissues along the GI tract of normal human and animals [83]. Using *in situ* hybridization and immunohistochemistry, small intestine showed abundant distribution of VDR from duodenum to ileum. Moreover, VDR expression was observed in normal colonic epithelial cells [84]. In stomach, isthmus region in corpus and scattered cells in pyloric antrum were identified as target tissues of ^3H-$1\alpha,25(OH)_2D_3$ [82] Columnar mucosa as well as Submucosal glands and ducts of the human gastroesophageal junction showed positive immunofluorescence to VDR [85].

Stating about cancerous tissue, VDR was poorly expressed in malignant gastric tissue [86] and Barrett's esophagus [85] when compared to normal tissues, suggesting

that loss of VDR activity is associated with malignancy. Interestingly, the non-malignant gastric tissues, premalignant gastric tissue and malignant gastric tissue were expressing VDR in different levels. For example, the moderately differentiated premalignant gastric tissues highly expressed VDR comparing to the poorly differentiated premalignant gastric tissue [86], [85].

Therefore, being aware of the normal distribution of VDR in normal gastric tissue might assist in diagnosing and assessing gastric cancer.

1.6 Mouse Gastric Epithelium

In human, the stomach is divided anatomically into three main parts, Fundus, Body and Pyloric antrum. These parts are existing in mouse as Forestomach, Corpus and Antrum respectively [87], [88]. The epithelial lining of the corpus and antrum are buildup of long tubular gastric epithelial glands, which consists of different epithelial cells [89].

The building block of the glandular part is the gastric gland. The corpus gastric gland has four unique regions named as Pit, Isthmus, Neck and Base, each one of these regions is consist of dynamic epithelial cells. For instance, in the Pit region, surface mucus cells which secret mucus are mainly located. The Isthmus region has stem cells which has the ability to differentiate and give rise to any of the corpus gland cells. Mucus neck cells which are also mucus secreting cells are located in the Neck region. Parietal cells which secret hydrochloric acid are well characterized rounded cells scattered in the Isthmus, Neck and Base region. Zymogenic cells (also known as chief cells) which secrete pepsinogen, and Enterochromaffin-like cells (ECL) which produce histamine are located in the Base region [87], [90].

The components of the antral gastric gland are surface mucus cells in the Pit region, deep mucus cells, gastrin producing G cells, somatostatin producing D-cells and antral stem cells which are located in the Base [91], [92].

All the above mentioned cells originate from the gastric epithelial stem cells and their distribution vary in the different parts of the stomach [89]. Therefore, the gastric glands in the corpus and antrum are histologically different as detailed in (Figure 1.5).

Gastric epithelial homeostasis is maintained by the gastric stem cells. These cells not only have the ability to differentiate and give rise to any gastric epithelial cell, but also continuously regenerate and renew the gastric epithelium [89], [93].

Although previous studies linked VDR signaling to Ca^{2+} activity and cancer progress, the normal function of VDR in the stomach and how it can be involved in gastric homeostasis is unrevealed.

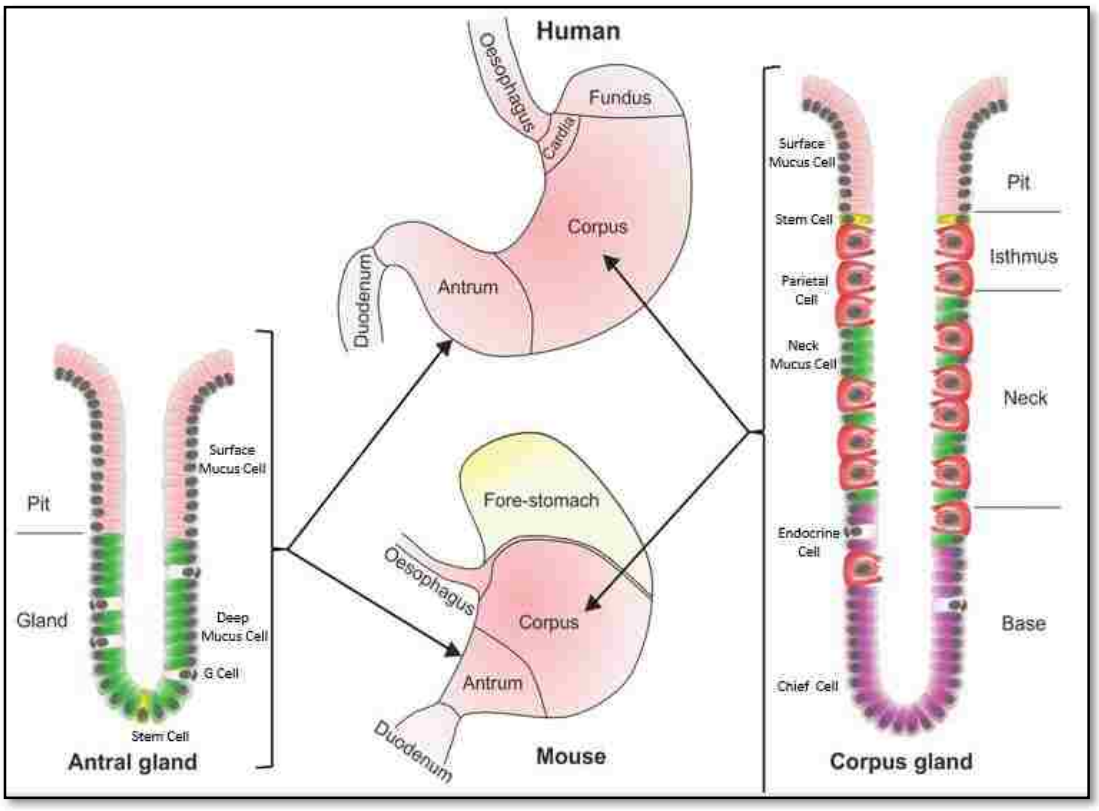

Figure 1.5: Mouse and human stomach anatomy.
The stomach consists of three sections, forestomach/fundus, corpus and antrum. The corpus and antrum are glandular units buildup of differentiated and undifferentiated cells (Modified from [94]). the corpus gastric gland is divided into 4 regions, Pit, Isthmus, Neck and base. while the antral gastric gland is shorter and consist of two regions; Pit and Gland.

1.7 Thesis Objectives and Hypothesis

Based on the literature review provided in early sections, the early discovery of vitamin D was after series of experiments linked to Rickets disease. However, late studies showed that vitamin D has substantial contribution at several biological activities.

As the intestine and other tissues in GI tract are expressing VDR, the expression and the role of this nuclear receptor in gastric epithelium is not well studied.

The goals of my Master Thesis were to identify the normal expression, distribution and cellular localization of VDR in gastric epithelium of C57BL/6J mice. Further, to study the expression of the enzymes (CYP2R1, CYP27A1, CYP27B1, CYP24A1) involved in vitamin D metabolism.

Since gastric epithelial homeostasis is maintained by gastric stem cells proliferation and differentiation, this thesis aims also to find the role of VDR in this process. This will be achieved by demonstrating the long term effect of low serum vitamin D on gastric epithelium VDR expression mice.

As the hypothesis was that VDR is involved in maintaining gastric epithelial cell proliferation and differentiation, Immunohistochemistry, RT-PCR and Real-Time PCR techniques were applied to support it.

Chapter 2: Methods

2.1 Animals

This study was approved by the Ethics committee of the United Arab Emirates University (UAEU). C57BL/6J mice were hosted in Specific Pathogen Free (SPF) environment at the animal facility of Faculty of Medicine and Health Sciences of UAEU.

After weaning at 3 weeks old, the mice were divided to 3 groups based on the diet and light exposure as the following:

Table 2.1: Diets and light/dark conditions

Group No.	**Diet**	**Light Cycle**
Group 1: (SDL)	Standard diet: Standard AIN-93G Rodent Diet with 1000 IU VD_3 (D10012Gi, Research Diet)	12 hr light/dark
Group 2: (SDD)	Standard diet: Standard AIN-93G Rodent Diet with 1000 IU VD_3 (D10012Gi, Research Diet)	No overhead light
Group 3: (VDD)	VD_3 deficient diet: AIN-93G Growing Rodent Diet with 25 IU VD_3/kg of diet (D17053003i, Research Diet)	No overhead light

The mice were kept in the previous conditions for 3 months, the weight was recorded on a weekly basis. The animals were fasted over night before dissection. Blood was collected for serum analysis and stomach tissue for IHC and Real-Time PCR.

2.2 Histological Analysis

The collected gastric tissues were fixed by Bouine fixative for 24 hrs, followed by 70% ethanol and then processed, paraffin embedded and sectioned (5 µm). After that the sections were deparaffinized and rehydrated for hematoxylin and eosin, immunoperoxidase staining or immunoflurescent staining. Slides were imaged using Olympus microscope IX83.

2.2.1 Immunohistochemistry

For immunoperoxidase staining, heat induced antigen retrieval was applied and then all the sections were pretreated with 3% H_2O_2 to inhibit endogenous peroxidase activity. The sections were incubated with 1% Bovine Serum Albumin (blocking reagent) for 1 hr. Rat polyclonal to anti-VDR antibody (ab115495, abcam, dilution 1:150) was incubated overnight at 4 °C and then biotin-SP conjugated goat anti-rat IgG (H+L) (112-065-003, Jackson immunoresearch, dilution 1:1000) was used at room temperature for 1 hr incubation. After PBS wash, sections were incubated with Extravidin-Peroxidase at dilution 1:1000 in blocking reagent at room temperature followed by another PBS wash. After that DAB: UREA (1:1) was added to sections for 6 min then washed twice using distilled water and counterstained with hematoxylin. The slides were dehydrated prior mountining with DPX.

Same protocol was follow for peroxidase staining of parietal cells using rabbit polyclonal to anti-H^+K^+ATPase-β primary antibody (ab176992, abcam, dilution 1:100) and 2 min incubation with DAB: UREA (1:1).

For 5-bromo-2'-deoxyuridine (BrdU) staining, mice were injected with 120 mg/kg BrdU (B5002, Sigma Aldrich) prepared in sterile distilled water. Intraperitoneal injection was given 2 hours prior sacrifice. The sections were incubated with mouse monoclonal to anti-BrdU antibody (MI-11-3, MBL Life Science, dilution 1:500) 1 hour at 4 °C and then followed by 1 hour incubation with biotin-SP conjugated goat anti-mouse IgG (H+L) (115-065-003, Jackson immunoresearch, dilution 1:500).

2.2.2 Multi-label Immunofluorescence

For co-immunofluorescence staining, PBS wash and permeabilization using 0.1% triton X-100 in PBS were performed after heat induced antigen retrieval. Then sections were incubated with 1% Bovine Serum Albumin (blocking reagent) for 1 hr and subsequently incubated overnight at 4 °C with anti-VDR primary antibody raised in rat (ab115495, abcam, dilution 1:150). Sections were washed with PBS three times for 5 min and then fluorescent goat anti-rat secondary antibody (112-165-003, Jackson ImmunoResearch, dilution 1:500) incubated at room temperature for 1 hr were used, followed by another PBS wash.

Parietal cell marker; rabbit polyclonal to anti-H^+K^+ATPase-β primary antibody (ab176992, abcam, dilution 1:100) was incubated along with anti-VDR primary antibody to detect the expression of VDR in parietal cells. While, lectins UEA-l-Rhodamine (RL-1062) and GSII (L21415, Life Technologies) were incubated with the fluorescent goat anti-rat secondary antibody for surface mucus cells and neck mucus cells detection, respectively. Secondary antibodies used were Cy3-conjugated goat

anti-rat IgG (H+L) (112-165-003, Jackson ImmunoResearch), Alexa Fluor 448-conjugated goat anti-rat IgG (H+L) (112-545-003) and goat anti-rabbit IgG H&L (TRITC) (ab6718, abcam).

Mounting media with 4′,6-diamidino-2-phenylindole (DAPI) for nuclei counterstaining was used and finally coverslip was placed. Negative control was prepared for each experiment by excluding the primary antibodies.

2.3 RNA Isolation

Total RNA was isolated from different parts of the mice stomach (forestomach, corpus and antrum) using RNeasy Mini Kit (Qiagen). RNA clean-up was performed using RNase-Free DNase set (Qiagen) as recommended by the manufacturer. To synthesize cDNA, 1µg of RNA was reverse transcribed using iScript™ cDNA synthesis kit (Bio-Rad).

2.4 Reverse-Transcription PCR

To study the expression of *VDR, CYP2R1, CYP27A1, CYP27B1* and *CYP24A1* in normal mouse gastric tissue, Reverse-Transcription PCR was conducted with the earlier synthesized cDNA using GoTaq Flexi DNA Polymerase kit (Promega) and using specific primers as detailed in table (2.1).

PCR product was separated by gel electrophoresis method using 2% agarose gel with Ethidium Bromide and visualized by Gel Doc™ EZ Imager (Bio-rad).

2.5 Quantitative Real-Time PCR

To study fold change in gastric differentiation, proliferation or VDR direct target genes, quantitative real-time PCR was performed using QuantiStudio® 5 Real-

Time PCR instrument (Applied Biosystems (ABI)). SYPER green dye was used for quantification of dsDNA after every cycle. Specific primers were used as shown in table (2.2). PCR reaction volumes of 20 µl were used with 40 amplification cycles. Each sample was tested in triplicate. Gene expression levels were calculated using the comparative cycle threshold method (ΔΔCT Method) and normalized to *GAPDH* gene expression level.

2.6 Serum Analysis for Vitamin D

Blood was collected immediately after sacrifice. Around 1 ml blood samples were collected in BD vacutainer (Refe. 367957) and centrifuged 3500 rpm for 15 min at 4°C for serum separation and stored in -80 °C. For Serum 25(OH)D analysis, total concentration of 25(OH)D ($25(OH)D_2$ + $25(OH)D_3$) was considered. Liquid chromatography tandem-mass spectrometry (LC–MS/MS) was used for measuring $25(OH)D_2$ and $25(OH)D_3$ concentrations. The LC–MS/MS system was comprised of a top of the range tandem mass spectrometer, model 8060, in combination with the Nexera ultra high-pressure liquid chromatography (UHPLC) system (Shimadzu, Japan).

Prior loading the samples to the LC-MS/MS instrument, extraction step was performed. Internal standard solution of $25(OH)D_3$ (6,9,9-D_3) was added to the samples. 1 ml hexane:ethyl acetate (9:1) mixture was used for extraction by adding it to the sample. The mixture was centrifuged and the organic layer was separated while the lower layer was further extracted twice by adding hexane:ethyl acetate (9:1) mixture. Calipratnts and quality controls were processed along the sample. They were all pooled together and dried at room temperature under sample concentrator. Finally, methanol/water (75:25, v/v) mixture was used to re-suspend the residue.

2.7 Gastric Acid Content Measurement

Stomachs collected from the mice on VD_3 deficient diet as well as standard diet were cut along the greater curvature. The stomachs were rinsed with 1 ml 0.9% NaCl (pH 7.0) and to collect clear supernatant the wash was centrifuged at 5000 rpm for 10 min. H^+ concentration was measured by doing manual titration; the supernatant against 0.005 M NaOH. The results were normalized to body weight (Kg).

2.8 Statistical Analysis

The statistical analysis was done using Graphpad Prism 7.0.3 to compare the different groups. The data was analyzed via one-way ANOVA test, Dannett's multiple comparisons post-test. Data was expressed as means ± SD. p values < 0.05 were considered as significant.

Table 2.2: Oligonucleotides primers for *VDR*, *CYP*s genes, gastric differentiation markers and some vitamin D_3 target genes

	Gene	Forward	Reverse	Product size (bp)
Receptor				
1	VDR	5'-CTCCATCCCCATGTGTCTTT-3'	5'-TTCTTCAGTGGCCAGCTCTT-3'	380
Vitamin D metabolites enzymes (CYP genes)				
2	CYP27B1	5'- TCAGCAGGCATCGCAGAAC-3'	5' GCATTGGATCCTGAGGAATGA-3'	77
3	CYP24A1	5'- CTCCCTATGGATGCAGTATGTATAGTG-3'	5'- TTTAAAAACGTTGTCAGTAGGTCATAACT-3'	102
4	CYP2R1	5'- CAGAAAGACGCTGAAAGTGCAA -3'	5'- CAGTGTATTTGTGTTTACTTGGCTTTATAA-3'	113
5	CYP27A1	5'- GGAGGGCAAGTACCCAATAAGA -3'	5'- TGCGATGAAGATCCCATAGGT-3'	88
Differentiation markers				
6	HK-α	5'-TGTACACATGAGAGTCCCCTT G-3'	5'-GAGTCTTCTCGTTTTCCACACC-3'	157
7	HK-β	5'-AAC AGA ATT GTC AAG TTC CTC-3'	5'-AGA CTG AAG GTG CCA TTG-3'	140
8	Gastrin	5'-GGACCAGGGACCAATGAGG-3'	5'-CCAAAGTCCATCCATCCGTAGG-3'	173
9	IF	5'-CTT GGC CCT GAC CTG TAT GT-3'	5'-TAG GTT GCT CAG GTG TCA CG-3'	191
10	Muc5AC	5'-GTGGTTTGACACTGACTTCCC-3'	5'-CTCCTCTCGGTGACAGAGTCT-3'	103
11	Muc6	5'-AGCCCACATTCCCTATCAGC-3'	5'-CACAGTGGAAGATTGCGAGAG-3'	192
VDR target genes				
14	PTHLH	5- GACGTACAAAGAACAGCCACTCA-3	5- TTTTTCTCCTGTTCTCTGCGTTT-3	81
15	TRPV6	5-TGACCCCTAAGGATGACCTCC-3	5-CTGTCCAAAGAATCGAGTGACC-3	122
16	P21	5-CGCTGTCTTGCACTCTGGT-3	5-CGTTTTCGGCCCTGAGATGTT-3	76
Housekeeping gene				
17	GAPDH	5'-TCAAGAAGGTGGTGAAGCAGG-3'	5'-TATTATGGGGGTCTGGGATGG-3'	350

Chapter 3: Results

Earlier studies identified the stomach as target tissue for 1α,25$(OH)_2D_3$by applying indirect method and using radioactive labeled vitamin D [82]. Although this study is indicating the responsiveness of stomach to vitamin D, the identity of gastric epithelial cell expressing VDR has not been illustrated yet in normal gastric tissue.

In this study, it was possible to characterize specifically the cells expressing VDR by following direct method and using specific antibodies for VDR and gastric epithelial markers. Additionally, it was possible to find out the possible role of VDR in maintaining gastric epithelial homeostasis by studying vitamin D deficiency by using vitamin D deficient diet.

3.1 VDR is Expressed in Normal Gastric Epithelial Tissues

To find out the normal distribution of VDR in the stomach parts, immunolocalization using VDR specific antibody were performed as demonstrated in (Figure 3.1).

The results showed a positive staining for VDR along the cells of corpus gastric glands and forestomach of these wild type mice. Nuclear staining as well as cytoplasmic staining was detected (Figure 3.1- E and F). The negative control showed no signal of DAP (Figure 3.1- C and D). Interestingly, not only the different parts of the corpus gastric gland: pit, isthmus, neck and the base were expressing VDR, but also the muscular part of the gastric tissue. It is worth mention that Stumpf *et al* illustrated earlier that cells in the isthmus region are selectively and weakly labeled with ^{3}H-1,25$(OH)_2D_3$ in rat gastric glands [82].

Furthermore, in agreement with the immunohistochemistry results, RNA analysis of forestomach, corpus and antrum using PCR and specific VDR primers confirmed that *VDR* gene is expressed in these different parts of the stomach (Figure 3.2).

3.2 Cytochrome P450 Genes (CYPs) Involved in VD_3 Synthesis and Degradation are Expressed in the Gastric Epithelial Tissue.

The expression of *CYP2R1*, *CYP27A1* and *CYP27B1*; Cytochrome P450 genes (*CYPs*) involved in VD_3 synthesis as well as *CYP24A1*; another Cytochrome P450 gene involved in breaking down of $1\alpha,25(OH)_2D_3$ had been analyzed in different regions of the gastric tissue of mouse (Figure 3.3).

RT-PCR detected expression of *CYP2R1*, *CYP27A1* and *CYP27B1* genes in forestomach, corpus and antrum of the wild type mice. Interestingly, expression of *CYP24A1* gene has been detected only in forestomach region of gastric mucosa.

The products size corresponded to the expected sizes of the CYPs genes amplicons which would have been produced with our primers (Table 2.2).

This great finding suggests that not only the stomach has the ability to synthesize the $1\alpha,25(OH)_2D_3$, but also it can degrade it when necessary.

3.3 Parietal Cells, Surface Mucous Cells and Mucous Neck Cells Express VDR

Numerous cells in the corpus gastric glands showed VDR expression with different patterns, nuclear and/or cytoplasmic as showed in (Figure 3.1). In order to investigate and specify gastric cells expressing VDR, co-immunofluorescence staining was performed using antibodies and lectins specific for gastric differentiated cells.

The parietal cells showed co-localization of anti-HK β antibody and anti-VDR antibody as shown in (Figure 3.4). This co-localization of VDR was nuclear as well as cytoplasmic. From this finding we can conclude that some parietal cells are expressing VDR.

Immunostaining of surface mucous cells using UEA lectin showed specific staining at the pit region of the corpus gastric glands (Figure 3.5-A). Additionally, we can see in Figure 3.5–B that VDR is expressed along the different cells which building up the corpus gastric glands. Therefore, to examine whether VDR is expressed in surface mucous cells dual immunofluorescence staining was performed. The results showed co-staining of UEA lectin and anti-VDR antibody in this type of cells (Figure 3.5-C), concluding that VDR is expressed in surface mucous cells.

Mucous neck cells were another corpus gastric gland cells that were assessed for VDR expression. GSll lectin, which specifically stain mucous neck cells in the gastric mucosa (Figure 3.6-A) as well as anti-VDR antibody (Figure 3.6-B) were double stained on the same tissue sections.

Immunoreactivity of VDR was detected in mucous neck cells, as there was co-localization of GSII and anti-VDR antibody (Figure 3.6-C).

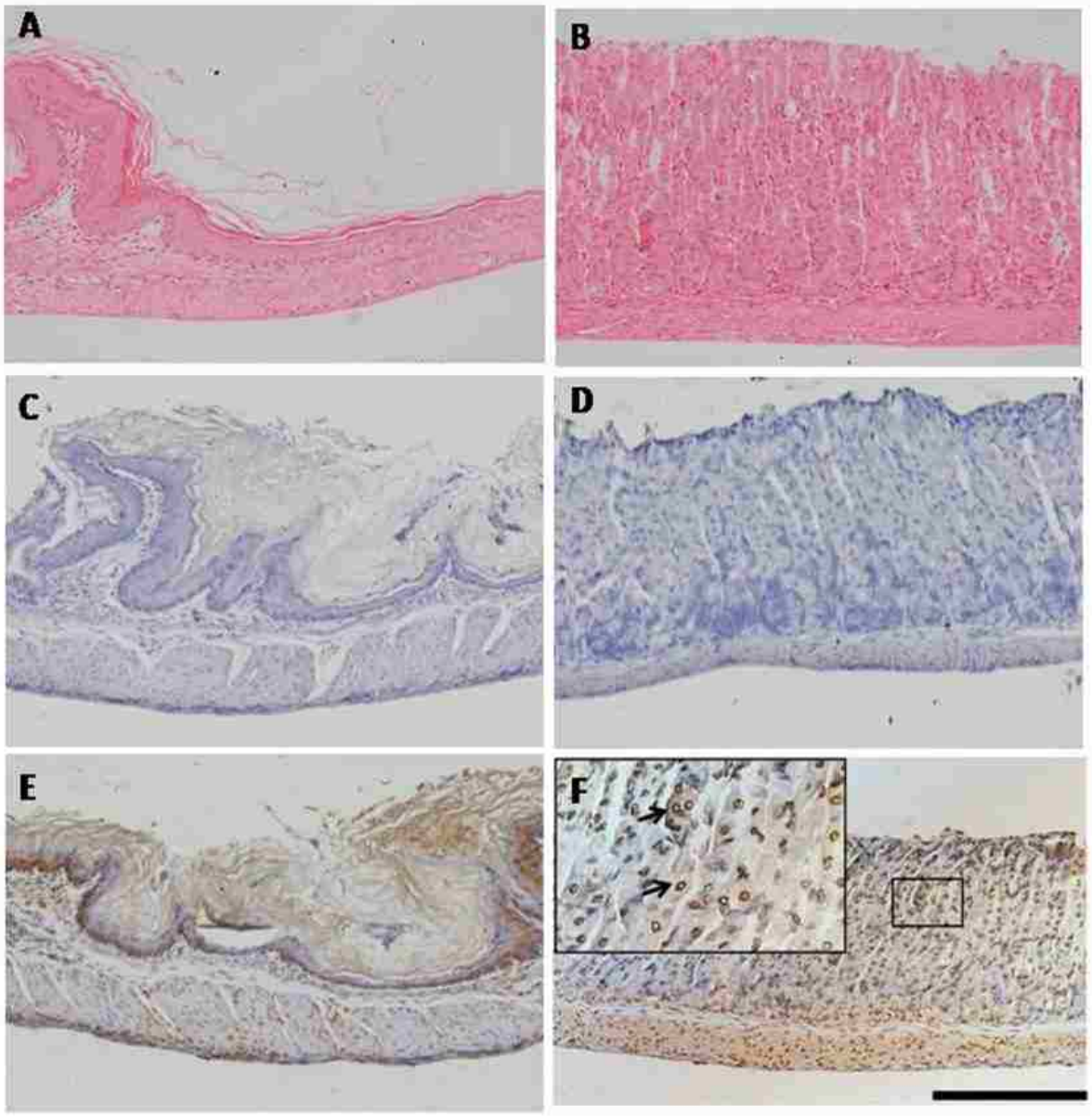

Figure 3.1:Immunohistochemical analysis of the corpus gastric glands showing Vitamin D Receptor (VDR) expression.
Wild type mice (age 2-4 months, n= 3) were sacrificed and stomach was collected. (A,C,E) The tissues were from forestomach and (B,D,F,G) corpus parts of the stomach of mouse. (A,B)H&E staining. immunoperoxidase staining using Rat polyclonal to anti-VDR antibody (dilution1:150, ab115495, abcam) and biotin-SP conjugated goat anti-rat IgG (H+L) (112-065-003, Jackson immunoresearch) (E,F,G). Scale bar: 200 µm.

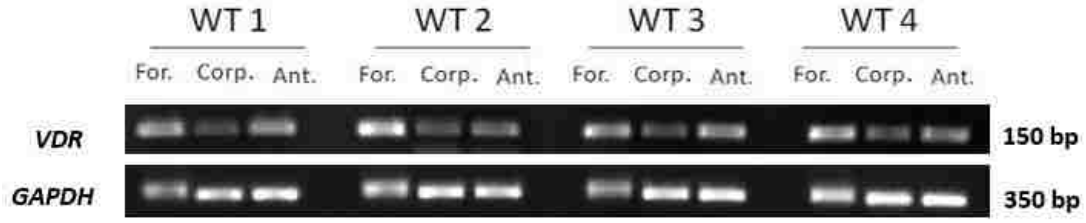

Figure 3.2: VDR mRNA is expressed in the gastric epithelial tissue of C57BL/6J wild type mouse.
RT-PCR detected expression of *VDR* gene in gastric epithelial tissue of C57BL/6J wild type mouse (age 2-4 months, n= 4). The tissues were collected from different parts of the stomach (Forestomach, Corpus, antrum). The three different parts of the stomach were expressing *VDR* gene.

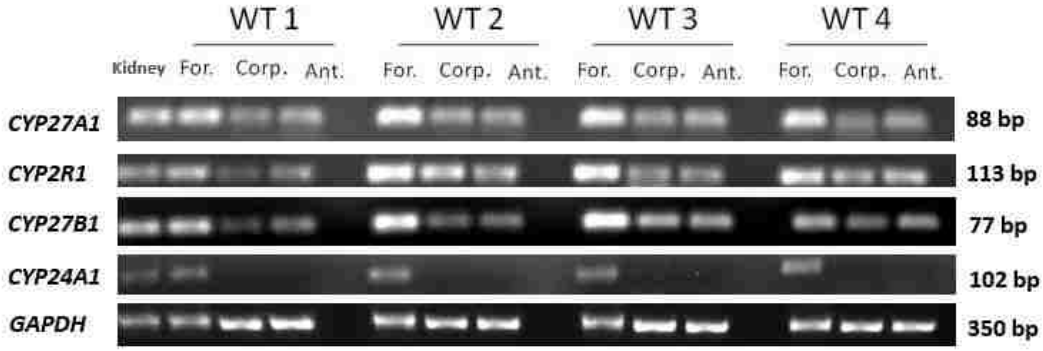

Figure 3.3: Cytochrome P450 genes (CYPs) are expressed in gastric epithelial tissue of C57BL/6J wild type mouse.
The tissues were collected from gastric epithelial tissue of C57BL/6J wild type mouse (age 2-4 months, n= 4) different parts of the stomach (Forestomach, Corpus, antrum). *CYP27A1*, *CYP2R1* and *CYP27B1* genes were expressed in the three different part of the stomach. While *CYP24A1* gene was expressed only in forestomach.

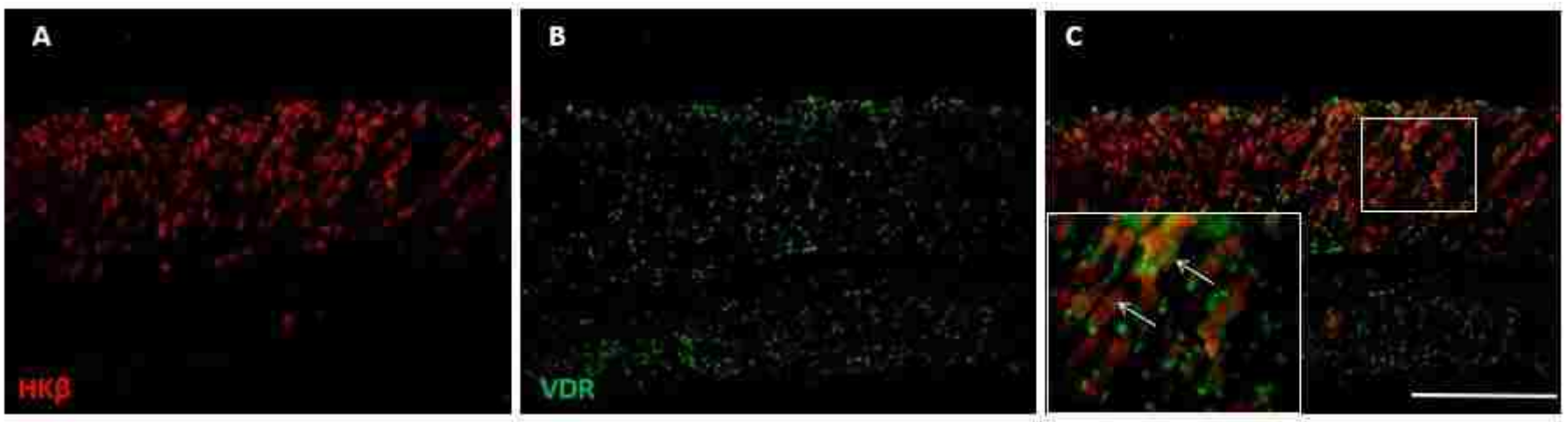

Figure 3.4:Parietal cells of corpus gastric glands are expressing VDR.
(C) Co-localization of anti-VDR and anti-HK β primary antibody in parietal cells of corpus gastric glands of C57BL/6J wild type mice (age 2-4 months, n= 3). The histological sections were co-stained with (B) anti-VDR antibody (ab115495, abcam, 1:150 dilution) and (A) anti-HK β primary antibody (ab176992, abcam, dilution 1:100). Scale bar: 200 µm.

Figure 3.5: Surface mucous cells of corpus gastric glands are expressing VDR.
(C) Co-localization of anti-VDR and UEA lectin in surface mucous cells of corpus gastric glands of C57BL/6J wild type mice (age 2-4 months, n= 3). The histological sections were co-stained with (B) anti-VDR antibody (ab115495, abcam, 1:150 dilution) and (A) lectins UEA-l-Rhodamine (RL-1062). Scale bar: 200 µm.

Figure 3.6: Mucous neck cells of corpus gastric glands are expressing VDR.
(C) Co-localization of anti-VDR and GSII lectin in mucous neck cells of corpus gastric glands of C57BL/6J wild type mice (age 2-4 months, n= 3). The histological sections were co-stained with (B) anti-VDR antibody (ab115495, abcam, 1:150 dilution) and (A) GSII lectin (L21415, Life Technologies). Scale bar: 200 µm

3.4 A Model of Vitamin D Deficient Mouse is Established.

To study the impact of VD_3 status on gastric epithelium, and gastric cells proliferation and differentiation, three groups of mice were fed different diets as detailed in (Table 2.1).

All diets were well tolerated by the mice. Serum levels of total 25(OH)D ($25(OH)D_2 + 25(OH)D_3$) were used to determine vitamin D status in these three groups of mice and reported in nanogram/millitre (ng/ml). Holick classification of vitamin D deficiency in human was followed [95]; vitamin D deficiency defined as 25(OH)D of <20 ng/ml, vitamin D insufficiency defined as 25(OH)D of 21-29 ng/ml and optimal level of 25(OH)D is >30 ng/ml.

Serum 25(OH)D analysis for SDL, SDD and VDD groups of mice showed that low dietary intake of VD_3 for 3 months reduced serum 25(OH)D from 33.14 ng/ml in the control group to 6.81 ng/ml in VDD group. Interestingly, our results demonstrated that vitamin D insufficiency (24.18 ng/ml) can be generated in mice by providing normal diet, but eliminating overhead light to prevent endogenous production; detailed in (Table 3.1).

Moreover, the weight of the mice was measured during the 3 months and weight gain was observed in VDD group compared to the control, however it was not dramatic change (p-value >0.05) (Figure 3.7). This finding was consistant with previous studies which linked vitamin D deficiency and overweight/obesity [96].

Additionally, the mice of the three groups did not suffer from hair loss, and the physical activity of all the mice was the same, including the mice which had been kept in dark.

Table 3.1: The effect of diets containing 1000 IU (standard diet) and 25 UI/Kg VD_3 (VD_3 deficient diet) on serum 25(OH)D concentration in mice.
Three groups of mice (SDL, SDD, VDD) (age ≈ 4 months) were fed different diet for a period of 3 months. Blood was collected after sacrifice and total serum 25(OH)D concentration ($25(OH)D_2$ + $25(OH)D_3$) was measured. Serum 25(OH)D concentration was represented as Mean (ng/ml) ± SD. One-way ANOVA test was used for data analysis. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Group of Mice	N	Mean (ng/ml)± SD	Vitamin D Status	P-value
Standard Diet /Light (SDL)	10	**33.14** ± 5.9	Normal vitamin D (25(OH)D > 30 ng/ml)	NA
Standard Diet /Dark (SDD)	10	**24.18** ± 7.4	vitamin D insufficient (25(OH)D of 21-29 ng/ml)	** P-value =0.0084
VD_3 Deficient Diet /Dark (VDD)	10	**6.81** ± 4.5	vitamin D deficient (25(OH)D of <20 ng/ml)	**** P-value =0.0001

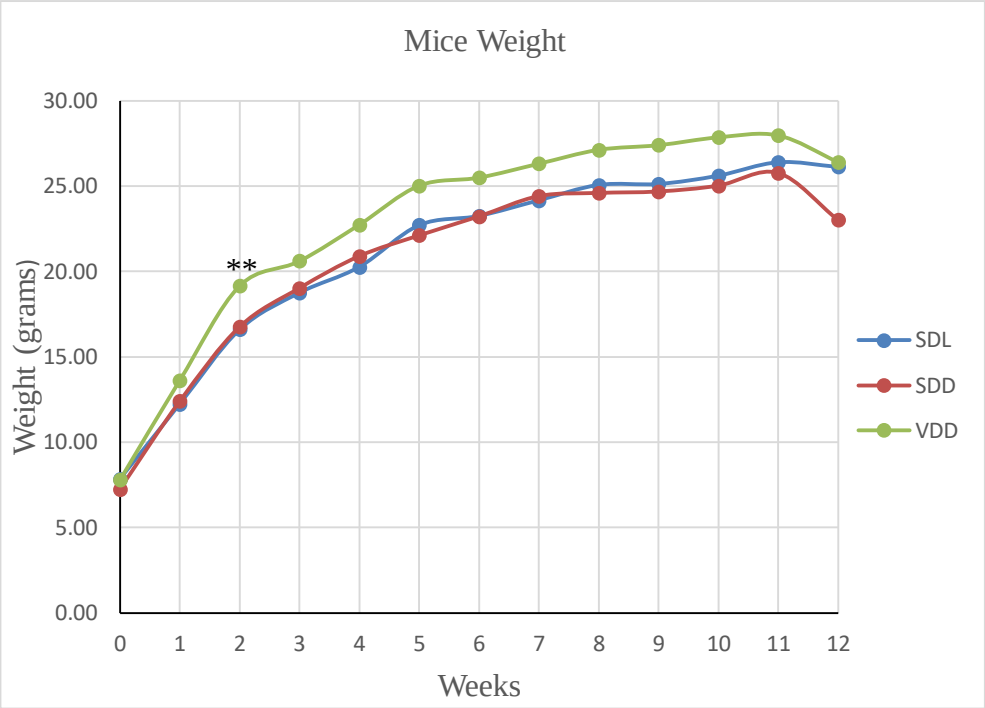

Figure 3.7: The effect of diets containing 1000 IU VD_3 (Standard Diet) and 25 UI/Kg VD_3 (Vitamin D_3 Deficient Diet) on mice weight.
Standard Diet /light group (SDL), Standard Diet /Dark group (SDD) and vitamin D Deficient group of mice (age ≈ 4 months, n= 7/group) were weighted regularly during 12 weeks. Vitamin D deficient mice gained more weight during the 12 weeks period compared to the control group. However, it wasn't significant. One-way ANOVA test was used for data analysis. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.5 Low Vitamin D Status Reduced Gastric Acid Secretion, Relative mRNA Expression of *HKα* and *HKβ* and Increase Gastrin Expression in Mice.

The above results indicated that VDR is highly expressed in normal stomach. So, it will be necessary to examine the effect of vitamin D deficiency on gastric target cells (Figure 3.8 and Figure 3.9).

Histological examination of gastric mucosa of the VDD, SDD as well as control group did not show any noticeable differences among the three groups of mice in-term of cells morphology, tissue length and overall tissue structure (Figure 3.8-A).

Moreover, parietal cells which are the acid secreting cells in gastric mucosa were studied. Microscopic examination did not show any morphological changes of parietal cells (Figure 3.8-B). However, when relative mRNA expression of HKα and HKβ were measured it was found that mRNA expression of the parietal cell marker dropped in a significant manner (p-value: 0.0001 and 0.0083 respectively) in vitamin D deficient group comparing to the control group (Figure 3.9-A-B).

Furthermore, H^+ concentration in gastric content collected from stomachs of fasting mice was measured (Figure 3.9-D). A reduction in H^+ concentration was observed, and consequently lower gastric acid content in vitamin D deficient mice was detected (p-value: 0.09) suggesting that low levels of serum 25(OH)D may reduce acid secretion in stomach. It is important to mention that the reduced gastric acid content in VDD group correlated with the reduced expression of HK subunits (proton pump).

It is well known that parietal cells secretion of acid is regulated by gastrin hormone [97], so we tested relative mRNA expression of gastrin in the different groups of mice. Expectedly, the relative expression of gastrin mRNA significantly increased (P-value: 0.0174) in vitamin D deficient group comparing to the control group. There

was an increment in gastrin mRNA relative expression in the group of mice kept in dark, however, this increment was not significant (p-value: 0.27) compared to the control (Figure 3.9- C).

3.6 Gastric Stem Cells Proliferation Increased in Vitamin D Deficient Mice.

To further understand the relationship between vitamin D status and stem cells proliferation in gastric mucosa of mice, BrdU staining was performed. BrdU incorporates with DNA during S phase, detecting newly generated cells.

BrdU labeled proliferating cells were counted by calculating the mean of BrdU positive cells in the corresponding areas of the gastric mucosa of the different groups of mice. An increased proliferation in gastric stem cells in the isthmus region of the corpus gastric gland was observed in vitamin D deficient mice compared to the control group (p-value: 0.004) (Figure 3.10). From this finding we suggest that low vitamin D status may increase proliferation rate of normal stem cells in gastric mucosa.

3.7 Expression of *VDR*, Gastric Differentiation Markers and 1α,25(OH)$_2$D$_3$ Target Genes in Normal and Low Vitamin D Level.

To check the regulatory effect of VD_3 on transcription of some genes such as *VDR, gastric* differentiation markers and some target genes of 1α,25(OH)$_2$D$_3$ (*PTHLH*, *p21* and *TRPV6*) in mouse stomach Real-Time PCR was conducted using RNA extracted from corpus.

In mice fed diet with low VD_3 level, VDR mRNA expression declined (P-value: 0.05) compared to the mice in control group (Figure 3.11-A). This result is expected since VDR expression is associated to endogenous production of 1α,25(OH)$_2$D$_3$ as some studies illustrated [98].

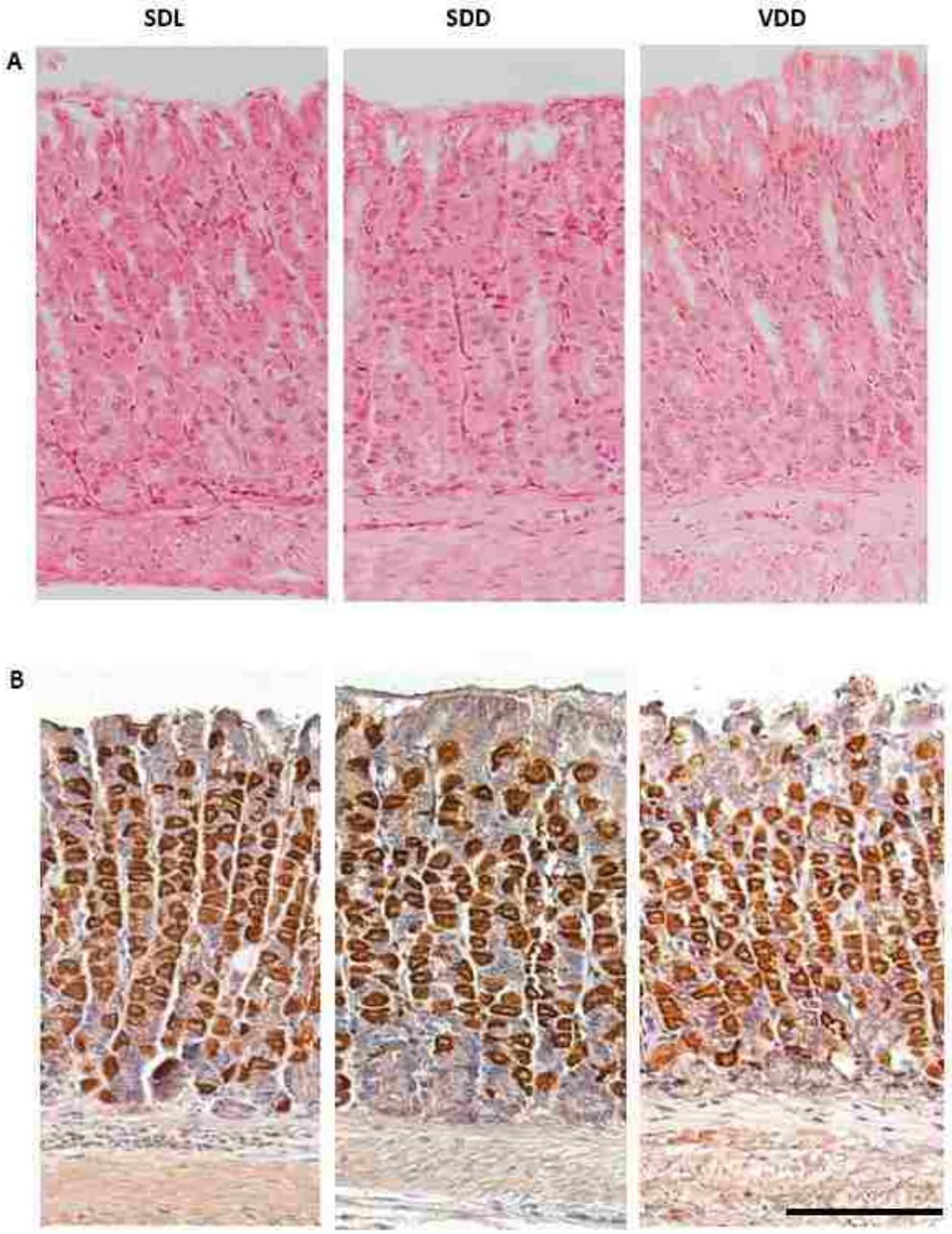

Figure 3.8: Immunohistochemical analysis of parietal cells showing that size and morphology didn't change with low status of 25(OH)D.
(A) H&E staining of gastric mucosa of SDL, SDD and VDD group of mice. (B) Peroxidase staining of parietal cells of SDL, SDD and VDD group of mice (age ≈ 4 months, n= 4/group) using anti-HKβ primary antibody (ab176992, abcam, dilution 1:100) shows no detection of parietal cells atrophy in vitamin D deficient mice. Scale bar: 200 µm.

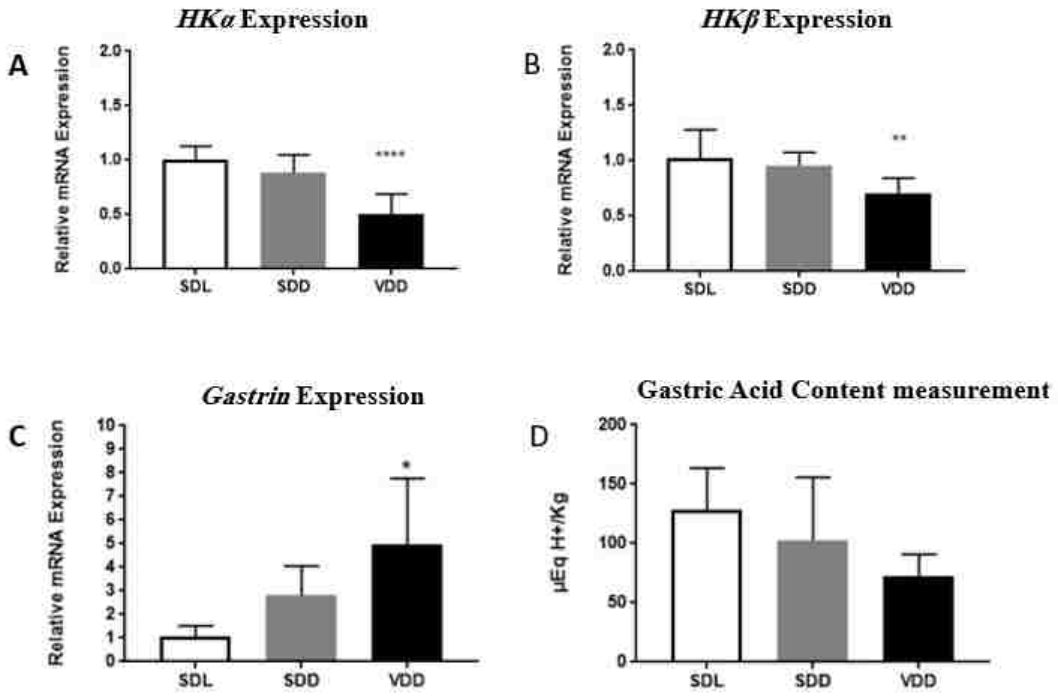

Figure 3.9: Low serum 25(OH)D concentration reduces *HK* sub-units gene expression and gastric acid secretion.
(A-C) Relative mRNA expression of HKα and HKβ significantly decreased, while expression of gastrin mRNA increased in VDD mice. (D) Gastric acid content decreased in vitamin D_3 deficient mice comparing to the normal mice. (age ≈ 4 months, n= 7/group). One-way ANOVA test was used for data analysis. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

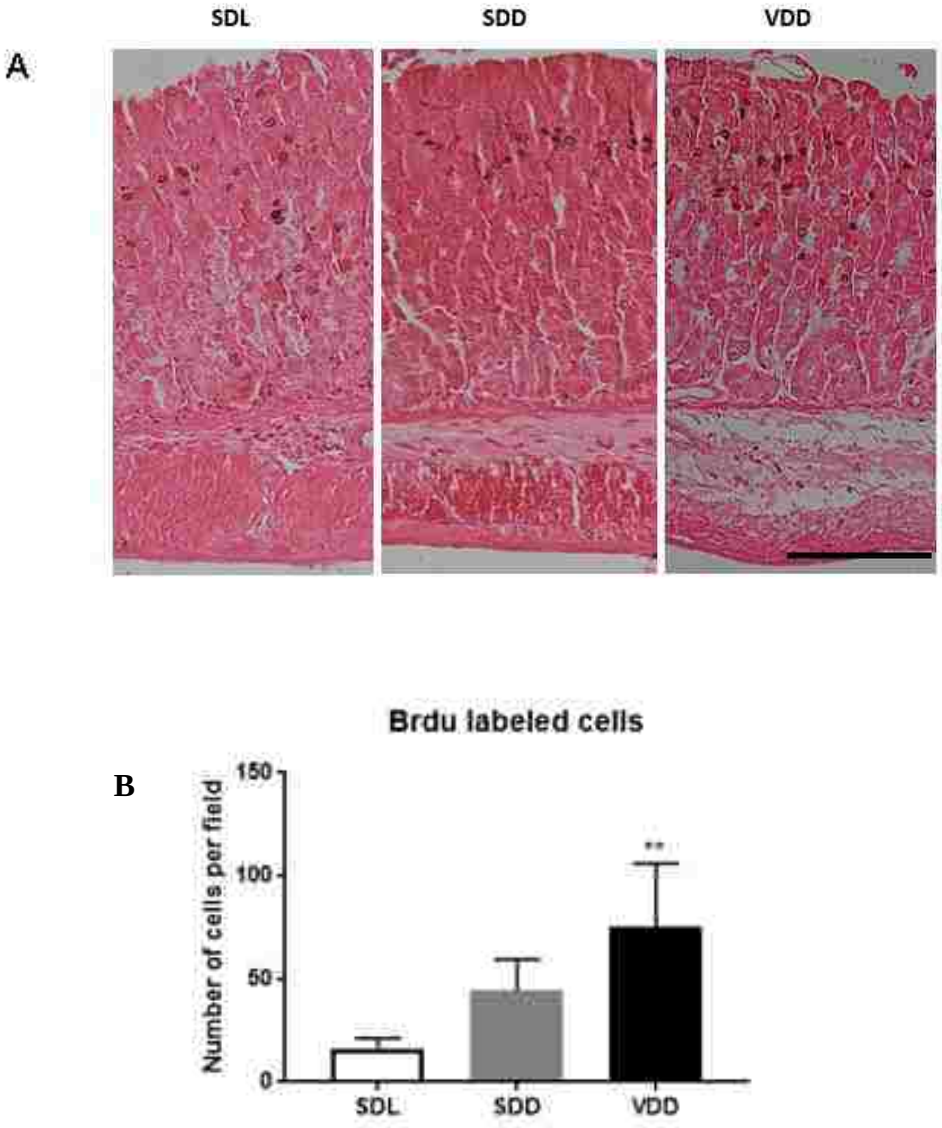

Figure 3.10: Gastric stem cells proliferation is increased with low serum 25(OH)D concentration in mice.
(A) Immunohistochemical analysis using anti-BrdU antibody (MI-11-3, MBL Life Science, dilution 1:500) shows BrdU labeled proliferating cells in isthmus region of corpus gastric gland of SDL, SDD and VDD groups of mice (age ≈ 4 months, n= 4/gorup). Scale bar: 200 µm. (B) Statistical analysis of BrdU labeled cells illustrate an increment in number of this cells. One-way ANOVA test was used for data analysis. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Next, we investigated mucus secretion in gastric epithelium in case of low and normal serum vitamin D level by studying the relative expression of *Muc5AC* and *Muc6* genes (markers for surface mucus cells and mucus neck cells respectively). Our results showed a very slight decrease in transcription level of these two genes in vitamin D deficient mice as well as control dark group compared to the control group (Figure 3.11-B&C). We suggest that low serum circulating 25(OH)D slightly decrease the secretory activities of surface mucous cells and mucous neck cells or their differentiation, and consequently do not impair the gastric mucus secretion.

Moreover, *IF* gene expression which is another gastric differentiation marker specific for chief cells, showed a remarkable decline (P-value: 0.0309) in the deficient group (Figure 3.11-D).

$1\alpha,25(OH)_2D_3$ regulates transcription of several genes through VDRE on the promoter of these target genes. *PTHLH*, *TRPV6* and *p21* are VD_3 target genes which are expressed in stomach [44], [52], [48]. When we compared relative mRNA expression of these genes in gastric mucosa of vitamin D deficient mice and the control group, we noticed a considerable decrease in *PTHLH* mRNA relative expression (P-value: 0.002), and a slight decrease in *TRPV6* and *p21* expression (P-value >0.05) in vitamin D deficient mice (Figure 3.12).

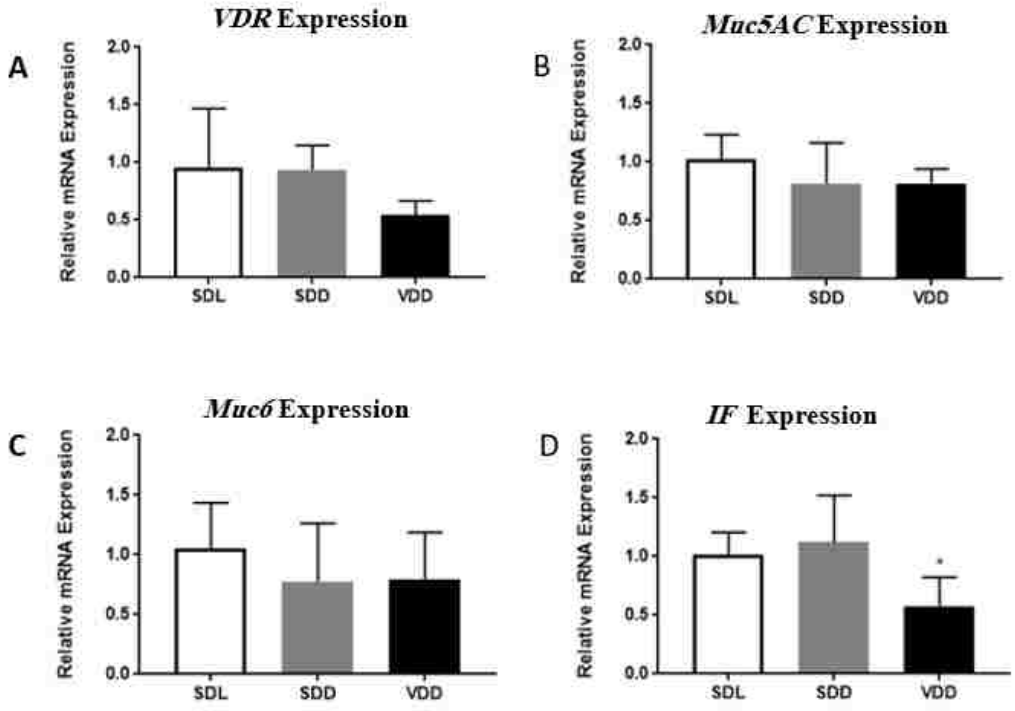

Figure 3.11: Representative relative mRNA expression of VDR and some gastric differentiation markers in normal and low serum 25(OH)D concentration in mice. RNA was extracted from corpus of SDL, SDD and VDD groups of mice (age ≈ 4 months, n= 7/group) and Real-Time PCR was conducted. VDR gene as well as the differentiation markers expression is decreased in vitamin D deficient mice compared to the control. One-way ANOVA test was used for data analysis. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

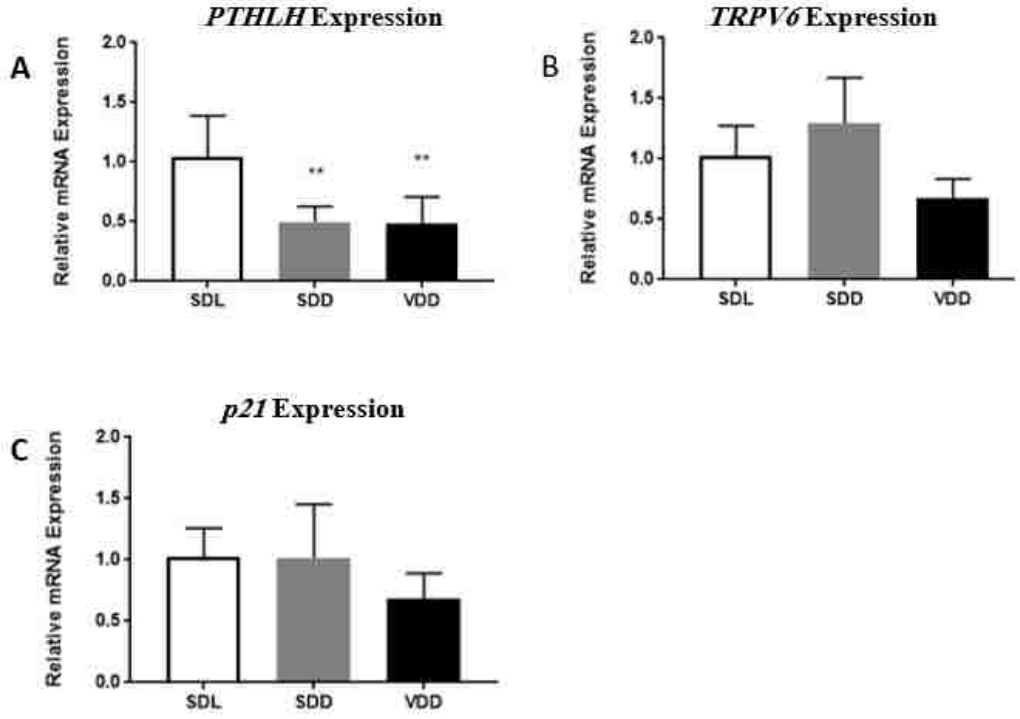

Figure 3.12: Representative relative mRNA expression of some $1,25(OH)_2D_3$ target genes in normal and low serum 25(OH)D concentration in mice.
RNA was extracted from corpus of SDL, SDD and VDD groups of mice (age ≈ 4 months, n= 7/group) and Real-Time PCR was conducted. Relative mRNA expression of PTHLH, TRPV6 and p21 are decreased in vitamin D deficient mice compared to the control. One-way ANOVA test was used for data analysis. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Chapter 4: Discussion

Several studies evaluated the physiological functions of VDR and VD_3 status in humans and rodents. Researchers demonstrated earlier that VD_3 is involved in calcium homeostasis [4], cell differentiation [62] and insulin secretion [78].

VDR is widely distributed in body tissues and organs. In gastrointestinal tract, small Intestine, colon and gastroesophageal junction are expressing VDR [83], [85]. Additionally, target tissues of $1\alpha,25(OH)_2D_3$ were detected in stomach [82]. The present study clearly showed expression of VDR in different parts of the gastric epithelium. Stumph *et al* demonstrated that only certain cells in rat's stomach such as mucous neck cells and chromaffin cells of the gastric glands are considered target cells of $1\alpha,25(OH)_2D_3$ using radioassay [82]. The results in this thesis showed a wide distribution of VDR along the corpus gastric glands by using immunohistochemistry method. Surface mucous cells, neck mucous cells as well as parietal cells exhibited localization of VDR.

Not only VDR expression was detected, but also *CYPs* genes which are involved in $1\alpha,25(OH)_2D_3$ synthesis and breakdown were found expressed in the different parts of the mouse stomach. We found that the corpus and antrum gastric glands are specifically expressing *CYP2R1*, *CYP27A1* and *CYP27B1*, the genes encoding for the enzymes responsible of producing the $1\alpha,25(OH)_2D_3$ [23], [27], [21], suggesting that these two parts of gastric epithelium have the ability to locally generate bioactive VD_3.

It was reported earlier that *CYP24A1* gene which encodes for the protein that inactivate $1\alpha,25(OH)_2D_3$ [31] is also regulated by $1\alpha,25(OH)_2D_3$ in negative feedback loop mechanism through VDRE on the promoter of *CYP24A1* [99]. Remarkably, this

study showed an expression of *CYP24A1* gene exclusively in forestomach (Figure 3.3). This finding indicates a significant role of forestomach in breaking down $1\alpha,25(OH)_2D_3$ when necessary and monitoring local VD_3 levels.

The second part of this study focused on investigating the impact of vitamin D deficiency on gastric epithelial cell differentiation and proliferation as well as gastric acidity.

It's well know that the ion pump HK of parietal cells plays a crucial role in gastric acid secretion by exchanging H^+ for K. Moreover acid secretion by parietal cells is regulated by gastrin hormone [100]. This study reports a decline in gastric acidity in vitamin D deficient mice along with lower mRNA expression of *HKα* and *HKβ* and increased *gastrin* mRNA expression. An interesting study carried by Antico and his team correlated between low serum concentration of $25(OH)D_3$ and Autoimmune Gastritis (AIG) among Italian population, and suggested hypovitaminosis D as risk factor for AIG [101]. AIG is caused by auto-production of antibodies against HK giving rise to inflammation in gastric mucosa, decreased gastric acid secretion as well as mucosal atrophy at the advanced stages [102].

Furthermore, our results showed a considerable increase in proliferation of gastric stem cells in vitamin D deficient group compared to the control (P-value: 0.004) (Figure 3.9). Similarly, other studies illustrated that rats fed VD_3 deficient diet for period of 3 weeks exhibited higher parathyroid cell proliferation compared to rats fed normal diet [103]. In another study, *VDR* knockout mice expressed a significant upregulation in Proliferating Cell Nuclear Antigen (PCNA) in the colon compared to the wild type mice [104]. Collectively, the results presented in this thesis as well as the other studies suggest the importance of vitamin D and VDR in regulating cell proliferation. Moreover, higher gastric stem cells proliferation in vitamin D deficient

mice might be also linked to higher gastrin expression in this group, since gastrin can stimulate cell proliferation [105].

By conducting Real-Time PCR, we tested the change in relative mRNA expression of some gastric differentiation markers such as *Muc5AC*, *Muc6* and *IF* as serum concentration of $25(OH)D_3$ changed in mice. Our results showed down regulation in all of these differentiation markers as $25(OH)D_3$ concentration decreased, however, the change was significant in *IF*. Concluding that the differentiation of surface mucous cells, mucous neck cells and chief cells was reduced due to serum $25(OH)D_3$ concentration reduction. These results were consistent with Driel *et a*l finding, when he stated that $1\alpha,25(OH)_2D_3$ can induce osteoblast differentiation as he was investigating the direct effect of $1\alpha,25(OH)_2D_3$ on osteoblast differentiation and mineralization in human osteoblast cell line (SV-HFO) [106]. In contrast to that, Chen *et al* showed an inhibitory role of $1\alpha,25(OH)_2D_3$ treatment on B cells differentiation to plasma cells. The expression of the key transcription factors involved in plasma cell differentiation didn't significantly reduced with $1\alpha,25(OH)_2D_3$ treatment. This finding suggested that $1\alpha,25(OH)_2D_3$ might have other means to suppress plasma cell maturation [5].

$1\alpha,25(OH)_2D_3$ and VDR can regulate transcription of target genes through VDRE on their promoter [44]. In the present study, the expression of *TRPV6*, *p21* and *PTHLH* have been examined.

TRPV6; Transient Receptor Potential Vanilloid member 6, is Ca^{2+} channel which is expressed in stomach and plays role in Ca^{2+} uptake in GI tract [48], [49]. *PTHLH* and *p21* are another $1\alpha,25(OH)_2D_3$ target genes which are expressed in stomach. PTHLH is a growth factor regulated by gastrin [107], while p21; Cyclin-Dependent Kinases Inhibitor is a tumor suppressor that cause cell cycle arrest [52].

The current results showed a slight decline in *TRPV6* and *p21*, while a significant decrease was reported in *PTHLH* expression in mice fed VD_3 reduced diet compared to control mice.

Pointing to the impact of low serum 1α,25(OH)2D_3 concentration on *TRPV6* expression, Walters *et al* elucidated the positive correlation between duodenum *TRPV6* expression and serum 1α,25(OH)2D_3 level in healthy men, regardless of the age. This finding suggested the effect of low 1α,25$(OH)_2D_3$ on intestinal calcium absorption. However, in healthy women, *TRPV6* expression strongly correlated with age [108]. On the other, Fleet *et al* reported constant intestinal *TRPV6* expression in mice fed low VD_3 diet (25 IU/kg) compared to mice fed normal VD_3 diet (1000 IU/kg). Disclaiming any association between *TRPV6* expression level and 1α,25$(OH)_2D_3$ concentration [109].

Another target gene is *p21*, which promotes cell cycle arrest [60]. *In vitro* study showed upregulation in p21 and VDR proteins expression in TMK1 cells (Gastric Adenocarcinoma cell line) when treated with 1α,25$(OH)_2D_3$. A reduction in cell number was observed as well. Suggesting anti-proliferative effect of 1α,25$(OH)_2D_3$ in *VDR* and *p21* dependent manner [110]. In our study, the noticeable upregulation of the proliferating cells only in vitamin D deficient mice might be related to the downregulation of *p21*, even-though this downregulation was not significant.

Mice fed VD_3 reduced diet showed considerable downregulation in relative mRNA expression of *PTHLH* in corpus tissue, while gastrin mRNA was upregulated. In contrast to our observations, Al Menhali *et at* found a regulatory role of gastrin on *PTHLH* expression. This regulation was evaluated by increased *PTHLH* mRNA abundance after treating wild type mice with gastrin and decreased *PTHLH* mRNA expression in gastrin-deficient mice [107]. Therefore, we are suggesting that there

might be other factors caused reduction in *PTHLH* expression in *VDR* and *gastrin* independent mechanism in vitamin D deficient mice.

Chapter 5: Conclusion and Future Directions

The present study aimed to investigate the normal expression, distribution and cellular localization of VDR in gastric epithelium of C57BL/6 mice. Further, to study the expression of the enzymes involved in vitamin D metabolism such as CYP2R1, CYP27A1, CYP27B1 and CYP24A1. Since gastric epithelial homeostasis is maintained by gastric stem cells proliferation and differentiation, we further aimed to study the role of VDR and vitamin D_3 in such mechanisms. These aims were achieved by performing different morphological and molecular techniques such as immunohistochemistry, RT-PCR and Real-Time PCR.

Our results showed i) the expression of VDR both in protein and mRNA levels in the forestomach, corpus and antrum parts of the gastric epithelium of wild type C57BL/6 mice, ii) the expression of the enzymes involved in vitamin D metabolism, and iii) vitamin D deficient mice were expressing lower *HKα*, *HKβ* and higher *gastrin* mRNA, consequently lower gastric acid content compared to the control mice. Therefore, it's very likely that vitamin D deficiency might increase susceptibility of having gastric disorders related to decreased gastric acidity. Additionally, acceleration in the proliferation of stem cells in isthmus region of the corpus gastric gland was observed in vitamin D deficient mice. By investigating the relative mRNA expression of some gastric differentiation markers such as *Muc5AC*, *Muc6* and *IF* in low serum vitamin D status, we found a slight decline in *Muc5AC* and *Muc6* expression and significant downregulation in IF. To sum up, our study provided data indicating that vitamin D and VDR are playing a considerable role in maintaining gastric epithelium differentiation and proliferation.

These approaches hopefully will improve the awareness regarding relationship between vitamin D deficiency and gastric diseases such as cancer and minerals absorption disorders. As we demonstrated a decline in tumor suppressor *p21* and calcium channel *TRPV6* mRNA expression in vitamin D deficient mice, we suggest to provide gastric cancer patients with vitamin D supplements as it has anti-proliferative effect to restrict tumor progression. Moreover, to prescribe vitamin D supplement to people suffering from gastric malabsorption of calcium due to vitamin D_3 deficiency.

In the current study, mice were fed VD_3 deficient diet for only 3 months, so this might be a limitation which gave rise to insignificant results in the expression some differentiation markers and target genes. Thus, to attain more chronic effect of vitamin D deficiency, extending the diet for period of 6 months to 1 year is recommended. Considering VD_3 deficient diet with high calcium (2%) and high phosphate (1.25%) (rescue diet) to avoid hypocalcemia and hyperparathyroidism. Furthermore, changing the mice strain to Nude mice might be an option, to allow more UV-B radiation penetration to the skin and eliminate hair as barrier in the control group. Additionally, it was discovered earlier that activation of VDR through $1\alpha,25(OH)_2D_3$ treatment in mice resulted upregulation in cholesterol 7a-hydroxylase (*CYP7A1*) mRNA and reduced level of cholesterol [111]. CYP7A1 is an enzyme which can metabolize cholesterol to bile acids in liver [112]. So, in future experiments, cholesterol can be measured in serum as well to study the correlation between cholesterol and vitamin D deficiency.

Moreover, to further study the mechanism of VD_3 and VDR action in maintaining gastric epithelial homeostasis (genetic or non-genetic), *in vitro* study can be conducted by treating mGEP cells with $1\alpha,25(OH)_2D_3$ and VDR antagonist (ZK159222); a vitamin D_3 analogue which has the ability to prevent VDR interaction

with the coactivators and consequently inhibit the genetic response [113], [62]. mGEP cell line; Mouse Gastric Epithelial Progenitor, is transgenic cell line established from the gastric mucosa of transgenic mouse expressing the simian virus 40 large T antigen gene in the parietal cell lineage. These cells will be very beneficial in understanding the possible role of vitamin D on gastric cell differentiation and proliferation [114].

Furthermore, to expand our findings and understand the role of vitamin D in regulating gastric stem cells proliferation, mGEP cells could be treated with different doses of $1\alpha,25(OH)_2D_3$ (10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M) and then cell viability test and cell count could be performed [115].

Moreover, we found in the present thesis that the different parts of the stomach are expressing the *CYPs* genes involved in vitamin D metabolism. So, to have better understanding of how the stomach can regulate $1\alpha,25(OH)_2D_3$ synthesis and degradation independently from liver and kidney, a further experiment including Real-Time PCR is recommended to check the relative mRNA expression change of *CYP2R1*, *CYP27A1*, *CYP27B1* and *CYP24A1* genes in the control group and VDD group.

Finally, in Dr. Asma's lab, we demonstrated the expression of VDR, Estrogen Receptor (ER) as well as Parathyroid Hormone 1 Receptor (PTH1R) in forestomach of wild type mice, so more study about this ignored tissue is highly important since little is known about it.

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Appendix

Supplementary table S.1: Representative table showing the different vitamin D metabolites detected by LC-MS/MS in serum of SDL group of mice.

S.NO	Sample type	volum(uL)	1,25(OH)2-D3 (ng/ml)	1,25(OH)2-D2 (ng/ml)	3-EPI-25OHD3 (ng/ml)	25OHD-3 (ng/ml)	25OHD-2 (ng/ml)	25OH(D2+D3) (ng/ml)	7,C4 (ng/ml)	VIT D3 (ng/ml)	VIT D2 (ng/ml)
1	SDl2	200	12	13	10.4	25	16	41	10	15	0
2	SDL3	150		10	12	22	6.5	28.5	26	24	15
3	SDL4	250			8	30	15	45	6	32	14
4	SDL5	300			3.5	15	10	25	21	40	
5	SDL7	150			16	21	11.4	32.4	19	20	5
6	SDL8	150			10	19	15	34	20	6	12
7	SDL9	150	8	8.5	15	27	7	34	6		45
8	SDL12	150	7	6	8	21	13	34			32
9	SDL13	150				15	10	25	5	30	24
10	SDL14	100	5	9	6.3	17.5	15	32.5	8.5		20
Mean	0	0	8.00	9.30	9.91	21.25	11.89	**33.14**	13.50	23.86	20.88

Supplementary table S.2: Representative table showing the different vitamin D metabolites detected by LC-MS/MS in serum of SDD group of mice.

S.NO	Sample type	volum(uL)	1,25(OH)2-D3 (ng/ml)	1,25(OH)2-D2 (ng/ml)	3-EPI-25OHD3 (ng/ml)	25OHD-3 (ng/ml)	25OHD-2 (ng/ml)	25OH(D2+D3) (ng/ml)	7,C4 (ng/ml)	VIT D3 (ng/ml)	VIT D2 (ng/ml)
1	SDD1	150	9	7	3.5	9.5	19	28.5	1.51		
2	SDD2	150			2.5	10	12	22	16	25	24
3	SDD5	150		9	10	16	8.5	24.5	3.5		40
4	SDD7	50			12	7	8.8	15.8	4		
5	SDD8	50				14	6.5	20.5	7.5		38
6	SDD9	150				17	21.5	38.5	22		
7	SDD11	150				16.5	3.5	20			21
8	SDD12	250			11	16		16		32	8
9	SDD13	150		10	6	12	24	36			23
10	SDD15	150			3	20		20	5.5		32
Mean			9.00	8.67	6.86	13.80	12.98	**24.18**	8.57	28.50	26.57

Supplementary table S.3: Representative table showing the different vitamin D metabolites detected by LC-MS/MS in serum of VDD group of mice.

S.NO	Sample type	volum(uL)	1,25(OH)2-D3 (ng/ml)	1,25(OH)2-D2 (ng/ml)	3-EPI-25OHD3 (ng/ml)	25OHD-3 (ng/ml)	25OHD-2 (ng/ml)	25OH(D2+D3) (ng/ml)	7,C4 (ng/ml)	VIT D3 (ng/ml)	VIT D2 (ng/ml)
1	VDD1	50				3.9	4	7.9			
2	VDD2	50				1.8	1.7	3.5			
3	VDD3	50				1.3	1.6	2.9	8		
4	VDD4	50			1.5	18		18			
5	VDD5	50				1.4	1.3	2.7			6
6	VDD6	50				3	3.6	6.6			
7	VDD8	50				2.6	0.8	3.4			5.1
8	VDD9	50				0.5	5	5.5	10	9	4
9	VDD12	50				3.6	2	5.6	7		12
10	VDD14	50				9	3	12	2		
Mean					1.50	4.51	2.56	**6.81**	6.75	9.00	6.78