DISCOVERING THE SPACIAL AND TEMPORAL COMPLEXITY OF INTESTINAL 1ALPHA,25-DIHYDROXYVITAMIN D ACTION

by

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ABSTRACT

The primary role of 1,25$(OH)_2$D and the vitamin D receptor (VDR) during growth is to mediate intestinal calcium (Ca) absorption by regulating the expression of genes (e.g., Trpv6, S100g) that control Ca fluxes through enterocyte. In contrast to the well-defined role during rapid growth, the understanding of 1,25$(OH)_2$D signaling in post-growth, mature adult or the elderly is poor. Some observational studies suggest intestinal 1,25$(OH)_2$D signaling is not important to Ca absorption and bone for mature adult. In the elderly, intestine develops resistance to 1,25$(OH)_2$D action which might be due to age-related reductions in intestinal VDR level. In addition, there is lack of evidence that directly tests the role of 1,25$(OH)_2$D action in the distal intestine post-growth. My dissertation research focuses on discovering the importance of 1,25$(OH)_2$D signaling from both the temporal and spatial perspectives. By using 4-month old, whole intestine or large intestine VDR knockout mice, we found that for mature mice, 1,25$(OH)_2$D signaling plays a minimal role in regulating Ca absorption and protecting bone mass when dietary Ca intake is adequate. In contrast, 1,25$(OH)_2$D signaling in the whole intestine, and to a lesser extent the proximal colon, is critical to upregulate Ca absorption and protect bone when dietary Ca intake is low. Next, we proved that the Ca absorptive machinery in the proximal colon can be locally stimulated to enhance the expression of Trpv6 by 1,25$(OH)_2$D released from glycoside and glucuronide forms of calcitriol. Furthermore, our transcriptomic analysis on 1,25$(OH)_2$D-regulated genes in the duodenum of mature (4-month old), middle-aged (11.5-month old), and old (20.5-month old) mice suggest although aging did reduce the induction of some genes by 1,25$(OH)_2$D, this effect is not universally present across the genome and it is not related to intestinal Vdr expression. The findings from my dissertation research serve as a foundation for future research to identify a) the potential of specifically targeting proximal colon to increase intestinal Ca absorption and protect bone in adult; b) the molecular mechanisms that contribute to the aging-associated, non-universal resistance to 1,25$(OH)_2$D action.

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

Intestinal calcium (Ca) absorption is the first step for food Ca to enter the body which then contributes to maintain whole body Ca homeostasis. Intestinal Ca absorption efficiency is associated with high peak bone mass in adolescents and reduced bone loss in adulthood (1-4). Both events are important for osteoporosis prevention. Intestinal Ca absorption is mediated by the active metabolite of vitamin D, 1,25 dihydroxyvitamin D ($1,25(OH)_2D$), and vitamin D receptor (VDR). The loss of $1,25(OH)_2D$ and VDR actions lead to significantly impaired Ca absorption and abnormal bone phenotypes during growth (5-7). However, intestine-specific transgenic VDR expression is sufficient to recover normal Ca absorption efficiency and bone growth in growing VDR KO mice (8). These experiments suggest that the regulation of intestinal Ca absorption is the single most important role for $1,25(OH)_2D$ and VDR signaling during growth.

While most research has studied Ca absorption in the proximal small intestine, emerging evidence shows that distal intestine plays a crucial role in the whole body Ca homeostasis. For instance, kinetics studies revealed that $1,25(OH)_2D$-mediated Ca absorption occurs at distal intestinal (9-11). Ca absorption was higher for patients with Crohn's disease when more of the large intestine is retained (12). On the contrary, Ca homeostasis was disrupted and bone microstructure was damaged with the loss of large intestine in rats (13, 14). Several studies have directly addressed the importance of $1,25(OH)_2D$ signaling in the distal intestine for the whole body Ca metabolism by limiting transgenic expression of VDR to the distal intestine in mice model (15). It should be noted that these studies were conducted in growing animal models, the role of distal intestine in adult Ca metabolism and adult bone health has not been tested.

The intestinal Ca absorption has also been reported to drop in adults (16-18) as their bone Ca acquisition and bone mineral density reach a plateau (19-22). Furthermore, $1,25(OH)_2D$-mediated adaptive increase in intestinal Ca absorption in response to dietary Ca restriction drops rapidly in mature rats compared to growing ones (3-week-old) (23). These findings suggest declined dependence of intestinal Ca absorption on $1,25(OH)_2D_3$ in adulthood. More interestingly, physicians observed that patients with hereditary vitamin D resistant rickets (HVDRR), who has dysfunctional VDR, can maintain normal serum Ca level and display no bone deformities in their

adulthood without the need for high dose of Ca supplementation(24, 25). Collectively, these evidence suggest that 1,25$(OH)_2$D and VDR signaling might not be important in intestinal Ca absorption and bone health for adult.

Advancing age has been associated with intestinal resistance to 1,25$(OH)_2$D actions, causing a suppressed increase in duodenal Ca absorption across a wide range of 1,25$(OH)_2$D concentrations in the elderly versus the young individuals (26, 27). Some evidence suggest that intestinal resistance to 1,25$(OH)_2$D actions is due to reduced VDR levels (28-30), however, others found no change of intestinal VDR level with aging (26). The identification of mechanisms that contribute to aging-induced intestinal resistance to 1,25$(OH)_2$D actions still warrants further research.

In this review, I will provide conceptual foundations for my dissertation research. First, I will provide an overview of the importance of intestinal Ca absorption in bone health during growth and in adulthood. Second, I will discuss thoroughly what is known about the critical role of 1,25$(OH)_2$D and VDR in mediating intestinal Ca absorption during growth and what the literature tells us regarding their role in distal intestine. Third, I will review the literature and discuss what we know about the role of 1,25$(OH)_2$D and VDR signaling in adult intestinal Ca absorption and bone health, and aging-associated intestinal resistance to 1,25$(OH)_2$D. In the end, research gaps will be identified and the overall aims of my dissertation research will be summarized.

1.2 Intestinal Ca Absorption is of Vital Importance to Whole Body Ca Homeostasis and Bone Health

Osteoporosis is a major health concern in the United States. It is estimated that more than 53 million people either already have osteoporosis or are at high risk for osteoporosis (31). Osteoporosis is characterized by low bone mass and bone structural deterioration, causing bone fragility and increased risk for fracture at the hip, spine, and wrist (32). Osteoporotic fractures are associated with significant morbidity, mortality, loss of independence, and financial burden (33). Therefore, developing strategies for osteoporosis prevention is of vital importance for reducing osteoporosis risk.

Bone metabolism has several phases throughout the lifespan. Initially, there is a phase of bone growth. Bone mineral accretion reaches a peak shortly after peak height gain (12.5±0.90

years in girls and 14.1±0.95 years in boys) (34) and 95% of adult bone mass is attained during the years of peak bone accretion and 4 years following the peak (19). Accumulating evidence suggests that peak bone mass (PBM) is a strong predictor of osteoporosis risk. A negative correlation between PBM and osteoporotic fractures was revealed through a mother-daughter study where daughters of women with vertebral fractures had significantly lower BMD than their age-matched controls (35). This suggests that abnormalities in BMD in women with fractures originate, in part due to a genetically programed acquisition of a low PBM. Therefore, maximizing PBM is critical to osteoporosis prevention. Reducing adult bone loss is another potential strategy for osteoporosis prevention. Significant bone loss occurs during the early 50s for both men and women. While men lose about 1% of their bone per year, in women this rate is increased to 2-4-fold higher due to the onset of menopause. Consequently, because women experience more bone loss, they are more likely to reach the threshold for osteoporosis (36). Therefore, both maximizing PBM and reducing adult bone loss are crucial for reducing osteoporosis risk.

There is evidence that high dietary Ca intake is necessary to attain high PBM and attenuate adult bone loss. The National Osteoporosis Foundation (NOF) recently conducted a review of randomized clinical trials since 2000. For instance, Dibba et al. (37) found that Ca carbonate supplementation at 1000 mg/d for 12-month in Gambian children (80 boys, 80 girls) increased bone mineral content (BMC) in the midshaft radius (4.6 ± 0.9%; $p<0.0001$) and in the distal radius (5.5 ± 2.7%; $p=0.042$). Ca supplementation is also associated with increased areal BMD (aBMD) over baseline in a co-twin study in which each pair (n=51) of females received either Ca carbonate (1200 mg/d) or placebo for 24 months (38). The benefits of Ca were observed at total hip (2.4% higher at 18 months); lumbar spine (1.0% higher at 12 months); and femoral neck (1.9% higher at 6 months). Thus, NOF released a position statement that gave Ca a grade of "A" based on the strong evidence supporting its role in the development of peak bone mass and bone mineral accrual (36). In addition to attaining higher bone mass during growth, a meta-analysis showed that Ca supplementation is also beneficial for osteoporosis prevention by reducing adult bone loss (39). Dawson-Hughes et al. (40) found that Ca supplementation attenuated bone loss at the spine, femoral neck and radius in late postmenopausal women whose habitual dietary Ca intake was low (< 400mg/d). Collectively, these clinical trials indicate that high Ca intake is essential for bone development during growth and for adaptation to varying bone loss during aging.

Intestinal Ca absorption is critical for bringing Ca from ingested food into the body. High Ca absorption has been linked to high peak bone mass in adolescents (41) (42). It is also positively correlated to femur BMD ($r=0.17$, $p=0.02$), trabecular bone volume BV/TV ($r=0.34$, $p<0.0001$), and trabecular thickness ($r=0.28$, $p=0.0004$) in a genetically diverse population of growing mice (43). In addition to benefit bone accrual during growth, high Ca absorption has been found to improve adult femoral aBMD ($r=0.20$, $p=0.03$) (3) and reduce the annual percent change in forearm BMC ($r=0.185$, $p=0.017$) (44). The intestinal Ca absorptive process is mediated by the active metabolite of vitamin D, 1,25 dihydroxyvitamin D ($1,25(OH)_2D$), and nuclear receptor, VDR. $1,25(OH)_2D$- and VDR-mediated intestinal Ca absorption not only occurs in the proximal small intestine, but also in the distal intestine (e.g., cecum and colon) (9) (10) (11). Studies utilizing mice model with transgenic expression of VDR specific to the distal intestine directly revealed this segment as a critical contributor to Ca metabolism and bone health (45) (15). Research on the capacity of Ca absorption in the distal intestine has significance in populations with compromised Ca absorption: patients with small intestine resection, menopausal women, etc. Therefore, this review focuses on the evidence regarding $1,25(OH)_2D$-mediated Ca absorption at the distal intestine and its contribution to whole body Ca homeostasis and bone health.

1.3 $1,25(OH)_2D$ is a Critical Regulator of Intestinal Ca Absorption

To identify and comprehend the importance of Ca absorption at the distal intestine to whole body Ca homeostasis and bone health, we must understand the physiology of Ca absorptive process in the intestine. Therefore, we will discuss the actions and molecular mechanisms of $1,25(OH)_2D$ and VDR that intestinal Ca absorption depends upon in the next two sections of this review.

In the early 20th century it became clear that vitamin D deficiency causes bone abnormalities. Nicolaysen and Eeg-Larsen (46) first characterized the bone changes caused by vitamin D deficiency such as rickets. Baylink et al. (47) later found vitamin D-induced rickets was caused by an enhanced bone resorption rate and a reduced bone formation rate. When vitamin D deficient rats were infused with Ca and phosphorus to compensate for the loss of intestinal Ca absorption and to maintain at serum Ca within normal ranges, histomorphometric parameters (e.g., epiphyseal growth plate thickness, trabecular osteoid volume) at tibiae were comparable to those treated with vitamin D (48). Similarly, 7 months of direct Ca infusion at 750-1000 mg/d also cured rickets in a

child (49). These studies suggest that a primary role for vitamin D in bone health is through its action on intestinal Ca absorption.

Nicolaysen first reported the dependence of intestinal Ca absorption on vitamin D in 1937 (50). Others have studied this in more detail. For example, intestinal Ca absorption efficiency reduced by 75% in vitamin D deficient rats (51). Similarly, hemodialysis patients with end stage renal disease, and therefore unable to make $1,25(OH)_2D$, had significantly lower net Ca absorption than those received $1,25(OH)_2D$ therapy (43 ± 31 mg vs 175 ± 40 mg, $p<0.005$) (52). Since then, the action of $1,25(OH)_2D$ in mediating intestinal Ca absorption has been carefully examined.

Vitamin D can be obtained from the diet or it can be synthesized from 7-dehydrocholesterol when the skin is exposed to UV-B light. Vitamin D from the skin or from the diet is hydroxylated to the prehormone 25 hydroxyvitamin D (25(OH)D) by the 25-hydroxylase (CYP2R1) in liver. Afterwards, the prehormone then hydroxylated to $1,25(OH)_2D$ by the 1α hydroxylase (CYP27B1) in kidney. This is the hormonally active form of vitamin D and it is produced and then released into the circulation to act as an endocrine hormone during times of need.

The physiologic role of $1,25(OH)_2D$ production is most clear during dietary Ca restriction. For example, when Favus and Haussler (53) fed rats a low Ca diet (0.002% Ca, 0.34% P, 2.2 IU vitamin D/g) for 3 weeks, serum $1,25(OH)_2D$ level was significantly higher in rats fed a normal Ca diet (181 ± 17 vs 27 ± 3 pg/ml). This caused absorptive Ca flux across duodenum to increase from 39 ± 20 to 187 ± 46 nmol/cm^2/h. The relationship among low Ca intake, renal $1,25(OH)_2D$ production, and increased intestinal Ca absorption efficiency has been reported in other animal studies (54) (55) (56). The positive correlation between serum $1,25(OH)_2D$ levels and Ca absorption efficiency has also been seen in a genetically diverse population of 11 inbred mouse lines ($r=0.35$) (43) and in older, free-living women ($r=0.35$-0.38) (57, 58).

The molecular actions of $1,25(OH)_2D$ are mediated through transcriptional events initiated upon binding to the VDR. The VDR was discovered in 1969 by Haussler and Norman (59). It is a member of the steroid hormone receptor superfamily of ligand-activated transcription factors (60). The binding of $1,25(OH)_2D$ to VDR induces the heterodimerization of VDR to retinoid X receptor (RXR), which then translocates from the cytoplasm to the nucleus. This $1,25(OH)_2D$-VDR-RXR complex regulates gene transcription by binding to vitamin D response elements (VDRE) at the promoters of target genes. The critical role of VDR in Ca metabolism and bone development is seen clearly in the case of hereditary vitamin D dependent rickets (HVDDR or type II genetic

rickets), a disease caused by a defect in the gene encoding VDR (5). HVDDR is characterized by short stature and alopecia (61) and can be modeled by VDR deletion in mice. For instance, VDR KO mice had 40% lower BMD than wild type (WT) and developed typical features of advanced rickets at weaning, including widening of epiphyseal growth plates, thinning of the cortex, and increased osteoid seams width (6). VDR KO mice also developed hypocalcemia (7.3±0.3 vs 10.1±0.3 mg/dL), hyperparathyroidism (182±34 vs 17±3 pg/mL), and elevated serum $1,25(OH)_2D$ (3634±268 vs 57±7 pg/mL) (7). More importantly for the purposes of this review, VDR deletion in mice also caused a 71% reduction (16.3±1.6% vs 55.4±3.8% in WT, $p<0.05$) in Ca absorption (62). However, VDR deletion-induced abnormal bone phenotypes and impaired Ca homeostasis in mice can be partially normalized by feeding a rescue diet containing 2% Ca, 20% lactose, and 1.25% phosphorus that bypasses the vitamin D-mediated transcellular Ca absorption pathway (63). Bone histomorphometric measurements (e.g., percent osteoid volume, osteoid surface), trabecular microstructure (thickness, number, separation), and width of the growth plate were all normalized in VDR KO mice fed the rescue diet (64). Furthermore, Xue and Fleet (8) directly tested the primary role of VDR in intestinal Ca absorption. They used a villin promoter-human VDR transgene to generate VDR KO mice with intestine-specific expression of human VDR (KO/TG). This was sufficient to restore intestinal Ca absorption in VDR KO mice. Whereas Ca absorption decreased by 53% in VDR KO mice, it was 26% higher than controls in KO/TG mice. As a result, transgenic intestinal VDR expression prevented the growth arrest, hypocalcemia, hyperthyroidism, and osteomalacia observed in VDR KO mice. Collectively, these evidence suggest that the regulation of intestinal Ca absorption is the single most important role for $1,25(OH)_2D$ and VDR signaling.

It is clear that intestinal Ca absorption depends on $1,25(OH)_2D$ and VDR signaling and the disruption of its regulation leads to substantially reduced Ca absorption and abnormal bone phenotypes. In the next section, we will further our understanding on this topic by discussing the molecular mechanisms underlying $1,25(OH)_2D$ and VDR upregulation of intestinal Ca absorption.

1.4 $1,25(OH)_2D$ Regulates Saturable Ca Absorption through Influencing Key Steps in Facilitated Diffusion Model

Intestinal Ca absorption follows both a saturable and a non-saturable pathway (Fig. 1.1). The relationship between these two transport pathways can be modeled mathematically by a Michaelis-

Menton-like equation that is modified to include a linear component. Saturable Ca transport (presumably transcellular) is prevalent in the proximal small intestine (duodenum and jejunum) (65) but it is absent in the ileum (66). It requires energy (67) for Ca extrusion across the basolateral membrane against the concentration gradient. Pansu and Bronner (66) found that saturable Ca absorption is regulated by $1,25(OH)_2D$. Saturable Ca absorption is absent in vitamin D deplete rats and the injection of $1,25(OH)_2D$ to these rats restored saturable Ca transport to a level comparable to vitamin D replete rats. In contrast to the saturable pathway, non-saturable Ca absorption (presumably paracellular) is a linear function of luminal Ca concentration (13% of luminal load per hour in humans) (68). Some studies found that $1,25(OH)_2D$ regulates this process through tight junctions proteins claudin 2 and 12 (69). This review will focus on the mechanisms underlying $1,25(OH)_2D$-mediated transcellular Ca absorption because it is mostly studied. A detailed discussion of the role of $1,25(OH)_2D$ in paracellular pathway can be found in the review paper by Fleet (70).

1.4.1 $1,25(OH)_2D$ regulates the key steps of facilitated diffusion of saturable Ca transport

Several models have been proposed for vitamin D regulated saturable Ca transport. This includes the facilitated diffusion, vesicular, and transcaltacia models (70). The facilitated diffusion model is the most studied model of the three. The foundation for this model was explained by Bronner et al. (71) based on their evaluation of existing kinetic and expression data. The facilitated diffusion model describes transcellular Ca movement in three steps: Ca transport at the apical membrane via transient receptor potential vanilloid type 6 (TRPV6), Ca across the enterocyte bound to calbindin, and Ca extrusion at the basolateral membrane via a plasma membrane Ca ATPase (PMCA) (Fig. 1.2). This section will discuss these three steps and the role that $1,25(OH)_2D$ plays at each step.

TRPV6 is a member of the transient receptor potential vanilloid superfamily of membrane proteins. TRPV6 was first cloned from rat duodenum and Peng et al. (72) identified it as the key mediator of apical transcellular Ca^{2+} uptake. TRPV6-mediated Ca uptake is voltage-dependent with a Michaelis constant of 0.44 nM. TRPV6 is highly specific for Ca and Ca uptake by TRPV6 doesn't seem to be coupled with other ions. TRPV6 protein consists of six transmembrane domains with a putative pore-forming region between domain 5 and 6 as well as N- and C-terminal domains (73). The molecular mechanisms for TRPV6 as Ca channels are not fully known yet. Saotome et

al. (74) suggested that Ca selectivity of TRPV6 depends on the aspartate side chains. Later, they also suggested that helical transition of the S6 helices induces conformational change and differential residues exposure which allow the opening of Ca channel (75). TRPV6 mRNA levels positively correlate to Ca absorption over a wide range of biological conditions, including dietary Ca restriction, vitamin D injection, or VDR deletion. Song et al. (55) found that after 7 days' consumption of a 0.02% Ca diet, plasma 1,25 $(OH)_2D$ level increased by 98%. This induces elevations in duodenal TRPV6 mRNA level by 83% and Ca absorption (to 66.7%) ((both correlate with diet-induced changes in plasma 1,25$(OH)_2D$ level (r^2=0.7, r^2=0.78, respectively, both $p<0.001$)). Exogenous 1,25$(OH)_2D$ injection (100 ng/100 g BW) in vitamin D deficient mice also causes increased duodenal TRPV6 mRNA level. Additionally, Van Cromphaut et al. (7) found that VDR KO mice showed a >90% reduction of duodenal TRPV6 mRNA level vs WT. These data suggest that TRPV6 is 1,25$(OH)_2D$ target gene that mediates this hormonal regulation of intestinal Ca absorption. However, the role of TRPV6 in 1,25$(OH)_2D$-mediated intestinal Ca absorption was challenged by studies using TRPV6 KO mice where active Ca transport still occurs at similar level to WT mice (76, 77). This suggests compensation by other Ca channels and other Ca binding proteins yet to be identified. However, TRPV6 KO mice has impaired mineralization under low dietary Ca intake, suggesting the essential role of TRPV6 with dietary Ca restriction. Furthermore, when Cui and Fleet (78) directly tested the role of TRPV6 in intestinal Ca absorption, they found that intestine epithelium-specific transgenic expression of TRPV6 increased intestinal Ca absorption, prevented low serum Ca and abnormal bone phenotypes associated with VDR KO. Therefore, although there may be compensatory mechanism in the KO mice, these findings indicate a direct role of TRPV6 in intestinal Ca absorption.

The fact that TRPV6 is 1,25$(OH)_2D$ target gene is also supported by more recent RNA-seq analysis of mouse proximal intestine, which showed upregulation of TRPV6 gene in response to exogenous 1,25$(OH)_2D$ treatment (79). Mechanistic study using chromatin immunoprecipitation (CHIP) assay revealed two vitamin D response element (VDRE) at the promoter region (-2.1 and -4.3 kb relative to the start site of TRPV6 transcription) of human TRPV6 gene that mediate intestinal response to 1,25 $(OH)_2D$ signaling (80).

The second step in the facilitated diffusion model is the diffusion of Ca across the enterocyte. Ca binding protein calbindin D is proposed to be the key mediator of intracellular Ca diffusion whose cellular level is the rate-limiting determinant of intestinal Ca absorption (71). Calbindin

D9k contains the EF-hand structural motif (helix-loop-helix) where the loop contains the oxygen ligands for the binding of two Ca ion (73). Observations using microscopic image showed that calbindin D9k acted as a diffusional carrier (ferry) that allows Ca to diffuse from apical to the basolateral membrane (81) (82). Consistent with the ferry role hypothesis, calbindin D9k mRNA and protein levels were directly regulated by $1,25(OH)_2D$ through VDR (31) (40). In contrast, findings from several other studies suggest that calbindin is not essential for intestinal Ca absorption. For example, cellular calbindin content remained high even after vitamin D-induced Ca absorption returned to basal rate in chicks (83) and in mice (55). Intestinal Ca absorption remained low even when calbindin level was elevated (62). Furthermore, Cui and Fleet (78) discovered the VDR-independent upregulation of calbindin D9k in TRPV6 TG-VDRKO mice. They believe that increased calbindin D9k acts as a buffer in response to elevated intracellular Ca^{2+}, not a mediator of Ca absorption. Therefore, whether calbindin D9k is a direct gene target of $1,25(OH)_2D$ in mediating the intestinal Ca absorption process is still unclear.

The third step of intestinal Ca absorption is Ca extrusion at the basolateral membrane. Wasserman et al. (84) identified the expression of PMCA1b as the Ca^{2+} pump that is localized at the basolateral membrane throughout the chick intestine. Similar to TRPV6, PMCA1b is also regulated by $1,25(OH)_2D$. $1,25(OH)_2D$ repletion upregulated PMCA1b mRNA level (85) as well as ATP-dependent Ca uptake across basolateral membrane in duodenum as well as ileum by 70% ($p<0.001$) (86). On the other hand, deletion of the isoform of PMCA1b (Atp 2b1) leads to impaired bone mineral density and responsiveness to $1,25(OH)_2D$ (87). Therefore, PMCA1b is a potential $1,25(OH)_2D$ target gene that mediates this hormonal regulation of Ca absorption. These accumulating evidence support the critical role of $1,25(OH)_2D$ in mediating the facilitated diffusion model of saturable Ca transport. A detailed discussion and review of the evidence regarding the role of 1,25 $(OH)_2D$ and VDR in alternative pathways is available in the review paper by Fleet (70). The next subsection is a brief summary of these alternative models.

1.4.2 $1,25(OH)_2D$ has been proposed to mediate saturable Ca absorption through alternative models

Vesicular model proposes sequestration of Ca into lysosome vesicles as the alternative mechanism to calbindin D as a Ca buffer during transcellular Ca transport (88). Luminal Ca is proposed to across the enterocyte by an endocytotic-exocytotic-vesicular flow process (89).

1,25(OH)D treatment increased the number (90), the cycling and activity of lysosomes (91), and the level of lysosomal Ca (89). When lysosomal Ca accumulation is prevented by lysosomal pH disrupting agents, vitamin D-mediated Ca absorption is blocked (89).

Different from the two models described above that require a prolonged period of $1,25(OH)_2D$ action, transcaltachia is rapid Ca transport that occurs within minutes of exposing the basolateral side to $1,25(OH)_2D$ (70). For instance, when chick duodenum was exposed to $1,25(OH)_2D$, Ca concentration in the serosal perfusate is increased by 40% within 14 mins (92). The rapid $1,25(OH)_2D$ actions are initiated at the cell membrane and are independent of new transcriptional events. $1,25(OH)_2D$ regulation of transcaltachia may be mediated by a membrane associated version of VDR (a novel, non-nuclear role of VDR) (93) or by a membrane associated rapid response steroid binding protein (MARRS) (94). However, the acceptance of the transcaltachia model as physiologically important pathway to Ca homeostasis has been limited because some key evidence is missing. For example, no serum elevation of $1,25(OH)_2D$ related to the occurrence of transcaltachia has been reported when $1,25(OH)_2D$ fluxes are essential regulators of Ca homeostasis. In addition, there is no direct evidence of the consequences of MARRS deletion on bone (50). Key evidence is warranted before the acceptance of alternative models as physiologically important pathways to Ca absorption and whole body Ca homeostasis.

The evidence we reviewed through the last two sections supports that intestinal Ca absorption depends on $1,25(OH)_2D$ signaling. This $1,25(OH)_2D$-dependent regulation of Ca absorption is demonstrated across various physiological conditions, e.g., dietary Ca restriction, $1,25(OH)_2D$ deficiency, $1,25(OH)_2D$ repletion, or VDR deletion. The background knowledge we learned from these two sections is the foundation of Ca absorption in the distal intestine because it also relies on $1,25(OH)_2D$ regulation. In the last section of this review, we will discuss key lines of evidence that reveal the existence of $1,25(OH)_2D$-mediated Ca absorption at the distal intestine and its contribution to whole body Ca homeostasis and bone health.

1.5 $1,25(OH)_2D$-mediated Intestinal Ca Absorption at the Distal Intestine is Crucial to Whole Body Ca Metabolism and Bone Health during Growth

Clinical studies in populations with compromised intestinal Ca absorption suggest that Ca absorption in the large intestine could be important for whole body Ca metabolism. For instance, people develop short bowel syndrome (SBS) that results from the removal of intestinal segments

to treat intestinal diseases (e.g., Crohn's disease), injuries or birth defects (e.g., necrotizing enterocolitis). SBS leads to nutrient malabsorption and impaired bone health (95). However, studies found that the consequences of SBS on nutrient absorption and bone health are attenuated by retaining more of the large intestine. Hylander et al. (12) evaluated Ca absorption among 62 patients with Crohn's disease who underwent small-bowel resection with at least 50 cm being removed. Among them, 20 also had their colon removed while 42 had functional colon. They found that Ca absorption was significantly lower in patients with ileostomy with colon removed than in patients with functional colon (10% vs 14%, $p<0.001$). Since then, more evidence has been discovered supporting the critical role of distal intestinal Ca absorption to whole body Ca metabolism and bone health. The focus of this next review section is to discuss key pieces of these evidence.

1.5.1 Evidence from animal models indicates that Ca absorption in the lower bowel is important for maintaining normal Ca metabolism and bone health

The removal of cecum in rats was associated with disrupted Ca homeostasis such as increased fecal Ca waste and reduced fractional Ca absorption (13). To compensate for cecum removal induced Ca absorption, active Ca transport was enhanced in the proximal colon with upregulated TRPV6 and calbindin-D9k levels. However, significantly reduced BMC, BMD were still observed at spine, tibia and femur. Further histological and bone microstructural changes in cecectomized rats were further evaluated by Charoenphandhu et al. (14). Deterioration in both trabecular and cortical bone phenotypes were observed, including reduced trabecular number ($p<0.01$), increased bone separation ($p<0.001$), reduced trabecular bone volume ($p<0.001$), and cortical thickness ($p<0.05$). These data suggest the crucial role of the cecum in body Ca homeostasis and bone health.

Recent evidence from transgenic and/or VDR KO mice model further supports the importance of distal intestinal Ca absorption to Ca mentalism and bone phenotypes. Xue and Fleet (8) studied the impact of transgenic VDR expression to the whole intestine of VDR KO mice on Ca absorption and bone phenotypes. Mice with intestine-specific transgenic expression of human VDR (HA-hVDR) were obtained using villin promoter. Compared with VDR+/- littermates, VDR KO mice were growth arrested when fed a standard chow diet containing 0.72% Ca. Their duodenal Ca absorption was 53% lower and duodenal calbindin D9k and TRPV6 mRNA levels were also 93%

and 99% lower, respectively. Consequently, VDR KO mice developed hypocalcemia with 44% lower serum Ca and hyperthyroidism with serum PTH being 84-fold higher than VDR+/-. Abnormal Ca absorption due to VDR deletion caused severe effects on bone phenotypes. Femur was shorter and BMD was reduced by 56%; femoral cortical area and trabecular area were reduced by 90% and 39% respectively. Growth plate is also disorganized and widened. However, abnormalities in Ca metabolism and bone phenotypes were normalized by intestine specific VDR expression. Growth arrest observed in KO mice were normalized in KO/TG mice. Intestinal Ca absorption was 26% higher in KO/TG mice than VDR+/- mice. Transgenic recovery VDR to the intestine increased calbindin D9k and TRPV6 gene by 150% and 67%, respectively. Serum Ca and PTH levels were also restored to similar level as VDR+/-. Femur length was normalized in KO/TG mice. Femur BMD was not only restored but increased by 14% in KO/TG than VDR+/-. Femoral cortical area and trabecular area also increased by 13% and 18%, respectively. Lastly, growth plate was normalized in KO/TG mice. This study shows that intestinal Ca absorption is the single most important role for VDR during growth. In contrary to this study, Marks et al. (96) found that proximal-intestine-specific VDR expression is insufficient to recover abnormal Ca metabolism associated with VDR deletion. They generated transgenic mice by expressing hVDR specific to duodenum and proximal jejunum of VDR-null mice using duodenal-specific enhancer fragment of the adenosine deaminase (ADA) gene. When mice (60-day old) were fed a normal Ca diet (0.95% Ca), serum Ca is significantly lower in VDR-null mice (6.1±0.7 mg/dL) vs WT (10±0.47 mg/dL). Transgenic expression of VDR specific to proximal intestine did not normalize this and serum Ca in hVDR+/VDR-null mice (7.1±1.2 mg/dL) is still significantly lower than WT. These data show that the capacity of the small intestine alone is insufficient to restore the phenotype of VDR KO mice. When coupled to the Xue and Fleet study (8) (which shows expression across the entire intestine can recover the phenotype), this suggests that vitamin D-regulated Ca absorption in the lower bowel must be contributing significantly to whole body Ca homeostasis during growth.

1.5.2 The machinery for vitamin D-mediated intestinal Ca absorption exists in the lower bowel

Cecum, ascending colon, and descending colon all demonstrate active Ca absorptive capacity. Early studies identified the kinetic characteristics of Ca absorption in each of these

intestine segments. They define mucosal to serosal Ca flux (J_{MS}) as absorptive while serosal to mucosal flux (J_{SM}) as secretory. Karbach and Rummel (9) found that in colon ascendants J_{MS} is significantly higher than J_{SM} (Vmax of 192 vs 93 nmol/cm^2/h, $p<0.01$), resulting in significant net Ca absorption capacity. They also found that 38% of J_{MS} is transcellular/facilitated diffusion while the rest is paracellular transport. While in colon descendants J_{MS} has a Vmax of 133 nmol/cm^2/h and this segment showed net secretory feature at Ca level >1.25 nM (97). Among all the distal intestinal segments, cecum has the highest rate of mucosal to serosal transport with a Vmax of 347.9 nmol/cm^2/h that is six times higher than the serosa-to-mucosa flux (10). Therefore, cecum is considered as the intestinal segment with the highest Ca absorption capacity. Among this absorptive Ca flux, 45% is active transcellular transport.

$1,25(OH)_2D$ has been shown to regulate active Ca absorption in these distal intestinal segments. For instance, to study the effect of $1,25(OH)_2D$ injection on colon active Ca transport, Favus and Mond et al. (11) injected $1,25(OH)_2D$ for 5 days (50 ng/day) to rats fed chow diet (1.2% Ca). $1,25(OH)_2D$ injection significantly increased J_{MS} (52.3±6.6 vs 31.7±4.2 nmol/cm^2/h for control, $p<0.025$) and led to greatly elevated net Ca transport (31.4±8.4 vs 10.6±4.8 nmol/cm^2/h for control, $p<0.05$) in descending colon. In addition, dietary Ca restriction enhanced J_{MS} (38.3±3.9 vs 25.6±1.4 nmol/cm^2/h) by inducing $1,25(OH)_2D$ signaling. This resulted in an increase in net Ca uptake (-0.2±2.0 vs 11.0±2.3 nmol/cm^2/h). Furthermore, Karbach and Feldmeier (10) studied the effect of $1,25(OH)_2D$ injection on cecum active Ca transport. Rats were fed a 1.1% Ca diet and simultaneously injected ethane-1-hydroxy-1,1-diphosphonate (EHDP) at a dose of 40 mg/kg to inhibit the 1α hydroxylase in the kidney. Because of $1,25(OH)_2D$ inhibition, EHDP reduced the J_{MS} to only 63% of the control (95.1 vs 151.1 nmol/cm^2/h, $p<0.0001$) and net Ca absorption was only about 50% of the control (62.7 vs 126.8 nmol/cm^2/h, $p<0.0001$). However, EHDP induced inhibition of the flux was restored (J_{MS}=181.7, Ca absorption=163.1 nmol/cm^2/h) by the concurrent exogenous $1,25(OH)_2D$ injection (250 ng/kg) to the level similar to control levels. Exogenous $1,25(OH)_2D$ also increased net Ca transport in human colon by performing steady-state vitamin D perfusion (2 µg/d for 1 week) in healthy subjects (n=10) (98). Lastly, expressions of key markers for vitamin D-mediated Ca absorption (e.g., TRPV6 and calbindin-D9k) are found not only in all segments of the small but also in the large intestine (8). These data suggest that active Ca absorption exists in the distal intestine and this process is regulated by $1,25(OH)_2D$.

1.5.3 Several studies have directly addressed the importance of 1,25(OH)$_2$D signaling in the distal intestine for Ca homeostasis and bone health

1,25(OH)$_2$D signaling is critical to Ca absorption in the distal intestine and this contributes substantially to whole body Ca homeostasis and bone health. To study the impact of VDR deletion from the distal intestine on Ca and bone metabolism, Reyes-Fernandez and Fleet (99) generated mice with VDR deletion specific to the distal intestine (ileum, cecum, and colon) using transgenic mice expressing Cre-recombinase driven by human caudal type homeobox 2 (CDX2) promoter that deletes floxed VDR alleles from the caudal region of the mouse (CDX2-KO). PCR analysis of mRNA expression revealed that CDX2-Cre driven VDR deletion is not only restricted to the large intestine but also extended to the kidney. CDX2-KO mice and control littermates were fed low 0.25% or normal 0.5% Ca diet since weaning for 7 weeks. Compared with WT, CDX2-VDRKO mice were smaller (-7%, $p=0.005$). Their TRPV6 mRNA and calbindin D9k level at proximal colon were also significantly reduced by >90% and >80% (both $p<0.001$) respectively. In response to the low Ca diet, serum 1,25(OH)$_2$D is elevated ($p=0.003$) in WT. But this adaptive elevation was lost in CDX2-VDRKO mice ($p=0.88$). Furthermore, CDX2-VDRKO had modest but significantly lower femur cortical area (-3.8%, $p<0.01$) and total area (-4.2%, $p<0.01$). However, different from expectations, serum PTH was 65% lower ($p<0.001$) in CDX2-VDRKO compared to control mice. And Ca absorption measured by oral gavage tends to be slightly higher in CDX2-VDRKO mice ($p=0.076$). This may be explained by the compensatory increase in Ca absorption in the small intestine that is evidenced by elevated duodenal VDR, TRPV6, and calbindin D9k mRNA levels in CDX2-VDRKO mice. This study supports that large intestine VDR significantly contributes to whole body Ca metabolism but duodenal compensatory mechanism may exist to minimize the negative impact of large intestine VDR deletion in growing mice.

Dhawan and Christakos et al. (15) then studied the impact of recovering VDR expression specific to distal intestine (ileum, cecum, and colon) to systemic VDR KO mice using CDX2 promoter. Three KO/TG mice lines (KO/TG line 1, 2, and 3) were generated by pairing VDR KO mice with TG mice. Mice were fed a standard chow diet from birth to 10- to 12-week-old. VDR transgene mRNA expression was confirmed by PCR analysis with the highest level found in the cecum and colon. However, KO/TG3 showed 50% lower VDR expression in the distal intestine than the levels observed in KO/TG1, KO/TG2, and WT. Growth arrest in VDR KO mice was prevented in KO/TG1 and KO/TG2 but not in KO/TG3. Elevations in serum PTH levels due to VDR deletion

were also reduced to the level of WT mice by recovering VDR to the distal intestine (for KO/TG1 and KO/TG2). Furthermore, bone defects associated with VDR KO such as widened, disorganized growth plate, reduced bone volume and cortical thickness were rescued in KO/TG1 and KO/TG2 mice. Transgenic expression of VDR (for KO/TG1 and KO/TG2) to distal intestine also normalized elevations in osteoid surface and thickness in VDR KO mice. This study supports the critical role of 1,25$(OH)_2$D signaling at the distal intestine in maintaining Ca metabolism related factors (e.g., PTH) and normal bone phenotypes in mice during growth and seems to suggest that the distal intestine is the most important segment. However, when coupled to the study by Reyes-Fernandez and Fleet (99) (which showed compensation from duodenum when VDR is deleted in the large intestine), these findings indicate that during growth, distal intestine plays a relatively less important role when the proximal small intestine has functional VDR, and its importance is more significant when the 1,25$(OH)_2$D signaling in the proximal intestine is compromised.

1.6 Evidence Suggest 1,25 $(OH)_2$D Signaling is Not Important in Adult Intestinal Ca Absorption and Bone Health

In the previous sections, we carefully discussed the role of 1,25$(OH)_2$D and VDR signaling in mediating intestinal Ca absorption and protect bone health during growth. In this section, we will review the available evidence on the role of 1,25$(OH)_2$D and VDR in adult intestinal Ca absorption and bone health and identify the research gaps in this area.

1.6.1 Basal and adaptive intestinal Ca absorption decline as individuals mature

As individuals mature, bone Ca deposition rate and dietary Ca requirement drop compared to the rapid growth period. Studies have shown that 95% of adult bone mass is attained during puberty and the immediately years following it. Afterwards, bone mineral acquisition ceased around 25-30 years old (19) (20, 21). The cease of bone gain is also observed in experimental mice model. For instance, BMD at vertebrae and tibia for C57BL/6 mice reached a plateau at around 16 weeks of age (22). Throughout adulthood, bone metabolism is in a balanced state (formation equals resorption) and bone mass is maintained under normal physiology conditions. Because of the reduced need for bone mineral acquisition, the recommended dietary allowance for Ca is 1000 mg/day for 19-50 years old adult men and women, which is 300 mg less than during growth (100).

Another change in Ca metabolism in adulthood is the decline in intestinal Ca absorption efficiency. The pubertal female has an average of 40% intestinal Ca absorption efficiency while it decreases to 26% in adult female (101). This age-related decline in intestinal Ca absorption is estimated to be 0.21% per year based on a longitudinal follow-up of 189 middle-aged (35-45 years old) women for 17 years (18). Not only in human, experimental animals also showed a decrease in Ca absorption efficiency from growth to adulthood. For instance, the net Ca absorption (total dietary Ca intake – fecal Ca output) is 36.7% for 7-week old young female rats while it reduces to only 9% in 4-mo old adult female rats ($p<0.05$) (26). Similarly, Song et al. (62) found that 2-month old mice fed a 0.5% adequate Ca diet has 55.4 ± 3.8% Ca absorption efficiency (in situ ligated loop) in their duodenum, which reduces to 18.0 ± 0.9% in 3-month old mice. Furthermore, the adaptation to low Ca intake also reduces in adulthood. A 10-day low 0.014% Ca diet treatment induced a 90% increase in Ca absorption efficiency (everted sacs) among 2-month old young male F344 rats. However, this adaptation declines to 60% for 4-month old and only 20% for 12-month old rats (23). These studies clearly showed a decline in basal as well as adaptive increase in intestinal Ca absorption in adult.

Because $1,25(OH)_2D$ signaling mediates intestinal Ca absorption during growth, people investigated the changes of $1,25(OH)_2D$ in adult and whether these changes contribute to the decline in adult intestinal Ca absorption. In 1982, Armbrecht et al. (102) studied the synthesis and catabolism of $1,25(OH)_2D$ in adult male F344 rats. They fed young and adult rats (4-week old and 12-month old) with vitamin D deficient diet (0.02% Ca and 0.6% P) for 4 wks. Then rats were randomized to receive thyroparathyroidectomy (TPTX) or Sham and received PTH or vehicle treatment following the surgery. In 4-week old young TPTX rats, PTH repletion increased renal $1,25(OH)_2D$ production by 61% and suppressed $24,25(OH)_2D$ production by 60%. In contrast, 12-month old adult TPTX rats showed no increase in $1,25(OH)_2D$ production in response to PTH treatment but $24,25(OH)_2D$ production reduced by 57%. This study clearly demonstrated a compromised $1,25(OH)_2D$ production in response to PTH stimulation in adult rats. They further assessed the response of vitamin D metabolism in adult rats under the condition of low Ca diet. They found that although low Ca diet induced an increase in PTH, which led to increasing renal $1,25(OH)_2D$ production and serum $1,25(OH)_2D$ level, these adaptive responses were minimal or abolished in adult rats, suggesting minimal dependence on $1,25(OH)_2D$ signaling-mediated adaptation to low Ca intake in adult (103). To further identify the mechanisms that contribute to

compromised renal 1,25$(OH)_2$D production in adult, Wong et al. (104) fed male Sprague-Dawley rats a 0.2% low Ca diet and a 0.6% adequate Ca diet. They found that the 1-month old growing rats had an 100% increase in renal 1,α -hydroxylase activity compared to those fed 0.6% adequate Ca diet. In contrary, the 4-month old adult rats didn't show any induction of 1,α -hydroxylase activity in response to the low Ca intake. Collectively, these studies showed the 1,25$(OH)_2$D signaling in adult do not respond to the stimulation of PTH and low Ca diet as well as it does during growth, which has been associated with reduced renal 1,α -hydroxylase activity in adult.

1.6.2 The importance of 1,25$(OH)_2$D and VDR signaling in adult intestinal Ca absorption and bone health is under debate

The decline in 1,25$(OH)_2$D synthesis and reduced response to PTH and low Ca diet stimulation in adult has raised the question whether 1,25$(OH)_2$D and VDR signaling is important to intestinal Ca absorption in mature, growth stable adult as it is for the rapid growth period. Some people believe they are not important for adult because of the clinical observations on hereditary vitamin D resistant rickets (HVDRR) patients who can maintain normal serum Ca level and bone phenotypes without functional VDR in their adulthood. HVDRR is a rare autosomal recessive disorder reported for the first time by Brooks et al. in 1978 (105). Symptoms of HVDRR start to develop in early infancy and they include alopecia, growth retardation, and skeletal deformities. In 1988, scientists discovered VDR gene mutations in patients with HVDRR (106). Since then, more has been discovered related to the underlying mechanism of this disease. For HVDRR patients, VDR gene mutations commonly seen in the ligand-binding domain and the DNA-binding domain of the VDR (107). The former can prevent the binding of 1,25$(OH)_2$D with the VDR, thus disables dimerization of the receptor-hormone complex with RXR and the recruitment of coactivators. The latter can disable the binding of the VDR-RXR complex to the VDRE of the target gene. Both abolishes the 1,25$(OH)_2$D-VDR transcriptional activity involved in mediating intestinal Ca absorption (108). Because of the compromised ability in absorbing Ca through VDR-dependent saturable route, HDVRR patients need to be treated with high dose of oral and/or intravenous Ca in order to maintain normal level of serum Ca and attain PBM during growth. Interestingly, in their adulthood, these individuals showed remission of the disease with normalization of serum Ca and showed no sign of bone deformities or fragility without additional Ca supplementation or treatment (24, 25). Because these individuals have permanent dysfunctional

VDR, their ability to maintain normocalcemia and healthy bone phenotypes suggest that VDR might not be essential for skeletal health in adulthood. However, it is important to know that dietary Ca intake requirement is met for some individuals reported in the clinical cases while it was unreported in other cases. Therefore, the conclusion cannot be drawn yet and the importance of $1,25(OH)_2D$ and VDR signaling in adult intestinal Ca absorption and bone health needs to be directly tested by future research.

1.7 Aging Induces Intestinal Resistance to $1,25(OH)_2D$ Actions

In the previous sections, we have discussed the critical role of $1,25(OH)_2D$ and VDR signaling in mediating intestinal Ca absorption and bone health during growth. We then reviewed current literature on their role in adult, which suggest adult might not require functional $1,25(OH)_2D$ and VDR signaling for their Ca absorption and bone health. In this section, we will focus on $1,25(OH)_2D$ metabolism in a different age group (the elderly) and discuss a physiologic phenomenon unique to this group, intestinal resistance to the hormonal actions of $1,25(OH)_2D$.

1.7.1 Aging-intestine shows resistance to $1,25(OH)_2D$ hormonal actions

More than 40 years ago, people observed reduced Ca absorption efficiency and the compromised ability to adapt to low Ca intake with advancing age (16, 109, 110). In 1965, Avioli et al. (109) tested the relationship between age and the intestinal Ca absorption by giving women (12-85 years old) a single oral ^{47}Ca test dose and their plasma ^{47}Ca concentration was measured as an indicator of the amount of Ca being absorbed. They found a negative correlation between the plasma ^{47}Ca activity (% dose per L) with age. The decline in basal intestinal Ca absorption was also observed in animal model. For example, Ca active transport measured by everted gut sacs from 3-week to 20-month old Sprague-Dawley rats was greatest at 3-wk, and it declined rapidly thereafter with age (23). The effect of age on intestinal Ca absorption was further assessed among young (22-31 years old) and older adult (61-75 years old) by feeding them with low Ca (300 mg/d) diet. Young adults adapted to the low Ca diet by increasing their intestinal Ca absorption by 66% ($p<0.05$), in comparison, older adults did not show a significant increase in Ca absorption. As the result of increased Ca absorption efficiency, the younger adults ended up absorbing a significantly higher amount of Ca (+45%) from the low Ca diet versus the older adults (110). All these studies

clearly demonstrated the decline in basal as well as adaptive increase in intestinal Ca absorption with advancing age.

Because $1,25(OH)_2D$ is the major hormonal regulator of intestinal Ca absorption during growth, people investigated whether the aging-induced decrease in Ca absorption is due to reduced serum $1,25(OH)_2D$ level. Several observational studies did find lower serum $1,25(OH)_2D$ level in the elderly. For instance, Gallagher et al. identified a negative correlation between Ca absorption and age ($r=-0.22$, $p<0.0025$), which is accompanied by significantly decreased serum $1,25(OH)_2D$ in the elderly (65-90 years old, n=10) subjects compared with the non-elderly (30-64 years old, n=34) subjects ($p<0.001$) (111). Similar observation on the negative association between serum $1,25(OH)_2D$ level and advancing age was also made in other studies on human and animal model (26, 112). In contrary, other studies found no change of serum $1,25(OH)_2D$ level with aging. When serum $1,25(OH)_2D$ level was measured among 194 healthy subjects at 15-90 years old, it remained the same across different age groups. Additionally, serum $1,25(OH)_2D$ level did not alter when it was measured on the same subjects (n=34, aged 70 at the start of the study) in the course of a decade (113). However, intestinal Ca absorption efficiency still fell with age when no change of serum $1,25(OH)_2D$ was detected, suggesting that factors other than $1,25(OH)_2D$ level causes Ca malabsorption with age (114).

Accumulating evidence indicates that aging-induced intestinal resistance to $1,25(OH)_2D$ is the contributing factor to Ca malabsorption in the elderly. The study by Eastell et al. (115) first observed intestinal resistance to $1,25(OH)_2D$ in the elderly. Opposite to the decline of serum $1,25(OH)_2D$ in other studies, they found that it increased with age (+38%, $r=0.40$, $p<0.01$) among 51 healthy women (26-88 years old). However, the increasing serum $1,25(OH)_2D$ did not increase the fractional Ca absorption with age. Similar observation on intestinal resistance to $1,25(OH)_2D$ was also made by others (30). But it was until 1998 that Wood et al. (26) first directly tested the effect of advancing age on intestine responsiveness to $1,25(OH)_2D$ action. Young (3-month old) and old (21-month old) male rats were infused with $1,25(OH)_2D$ at 14 ng/100g body weight/day for 3 days. $1,25(OH)_2D$ infusion raised plasma $1,25(OH)_2D$ in old rats (396 pg/ml) to similar levels as the young rats (391 pg/ml). However, the increase in duodenal Ca absorption efficiency measured by in situ ligated loop is only 1/3 in the old versus the young rats (7%, 19% Ca dose/10cm/10min, respectively). They then further examined the effect of age on the relationship between $1,25(OH)_2D$ level and intestinal Ca absorption across a wide physiological range of

$1,25(OH)_2D$. When this association was fitted in a linear regression model with plasma $1,25(OH)_2D < 400$ pg/ml, the slope of the regression line for the young rats was 0.13 while that of the old rats was 0.06 (0.06/0.13=0.46). This finding indicates that the intestinal response to increments in plasma $1,25(OH)_2D$ levels in old rats is only half of that in the young rats. This study directly proves that age causes intestinal resistance to $1,25(OH)_2D$ actions and impairs its induction of Ca absorption. Later on, the effect of age on the responsiveness of intestine across a broad range of circulating $1,25(OH)_2D$ was also tested in human subjects. Pattanaungkul et al. (27) manipulated the wide range of $1,25(OH)_2D$ levels by feeding the young (28.7 ± 5.3 year) and the elderly (72.5 ± 3.0 year) women with either high Ca, normal Ca, low Ca, low Ca plus 1µg/day $1,25(OH)_2D$, or low Ca plus 2µg/day $1,25(OH)_2D$. Similar to the findings by Wood et al. (26), they found that the change in fractional Ca absorption with increments in $1,25(OH)_2D$ in the elderly women is only 50% of that in the young women (slope ratio=0.5), indicating that elderly women have an impaired intestinal response to $1,25(OH)_2D$. Collectively, these studies clearly demonstrated reduced responsiveness to intestinal $1,25(OH)_2D$ action in the aging intestine.

1.7.2 Aging-induced intestinal resistance to $1,25(OH)_2D$ might be due to reduced intestinal VDR level

Because the molecular action of $1,25(OH)_2D$ is mediated via VDR, people then hypothesized that the intestinal resistance to $1,25(OH)_2D$ in the aging intestine may be due to reduced intestinal VDR level. The total and unoccupied VDR receptor (fmol/mg protein) in isolated duodenal epithelial cells from male Wistar rats decreased by 22% and 16%, respectively in 24-month old ones compared to 6-month old ones(28). The occupied VDR calculated as the difference of total and unoccupied receptor also fell significantly (-44%, $p<0.01$). Horst et al. (29) also found reduced unoccupied VDR receptor level in the small intestine of 20-24 month old male Fischer 344 rats compared to the 1-mo old control. In addition to animal model, human subjects also showed decreasing intestinal VDR protein level with age ($r=-0.38$, $p=0.03$) (30). However, others have shown opposite results regarding VDR level in the aging intestine. For example, Wood et al. (26) found that although plasma $1,25(OH)_2D$ level, duodenal calbindin D9k concentration and net Ca absorption (total dietary Ca intake – fecal Ca output) all declined in 17-month old female rats compared to 7-week old ones, there is no age-related change in total VDR receptor. The level of unoccupied VDR receptor also didn't change with age, which is opposite to findings from previous

studies using the same ^{3}H-1,25(OH)$_2$D binding assay (28) (29). These evidence we discussed above either support or dispute the hypothesis that low VDR levels impairs the intestinal responsiveness to1,25(OH)$_2$D, therefore, formal testing of this hypothesis is needed to resolve the inconsistency.

To evaluate the effect of reduced tissue VDR levels on Ca metabolism in the complex setting of whole-body physiology, Song and Fleet studied 90-day old young heterozygous VDR KO mice (HT) who have only half of the intestinal VDR content of WT mice. They evaluated the response of intestinal Ca absorption and gene expression along the time course of a single 1,25(OH)$_2$D dose (2 ng/g BW) and dose response to 1,25(OH)$_2$D (0.25, 0.5, 1 ng/g BW). In the time course experiment, no difference in 1,25(OH)$_2$D-induced increase in duodenal Trpv6 or calbindin D9k mRNA was found between HT and WT mice. In the dose-response study, there was also no difference in the expression of these genes at any dose of 1,25(OH)$_2$D between HT and WT mice. But the increase in duodenal Ca absorption efficiency (in situ ligated loop) in response to increasing 1,25(OH)$_2$D level was significantly higher in the WT mice versus HT, possibly due to increasing duodenal calbindin D9k protein level. However, it is important to know that 1,25(OH)$_2$D-induced intestinal Ca absorption in these young HT mice is still about 61% (slope ratio=0.61) of that in WT, which is higher than what was observed with advancing age in previous studies (26, 27). These findings indicate that there might be other mechanisms in addition to reduced VDR level that underlies aging-induced intestinal resistance to 1,25(OH)$_2$D, which need to be identified in future research.

The mechanisms involved in the execution of the molecular actions of 1,25(OH)$_2$D signaling through VDR could be possible contributors to the intestinal resistance to 1,25(OH)$_2$D with aging. These mechanisms include but not limited to the interaction between VDR and RXR, the binding of VDR/RXR complex to VDRE of the target gene, and the recruitment of co-activators (e.g., CREB/p-300, SWI/SNF). For instance, studies that investigate advanced renal failure associated 1,25(OH)$_2$D resistance in osteoclasts and monocytes revealed reduced nuclear localization of VDR (116) and binding of VDR/RXR complex to VDRE of the target gene (117). More research is needed to discover the mechanisms underlying aging-associated intestinal resistance to 1,25(OH)$_2$D actions.

1.8 Aims for Dissertation Research

Our review of the literature has shown the critical role of $1,25(OH)_2D$ and VDR signaling in mediating intestinal Ca absorption and protect bone health during growth. In addition to the proximal small intestine, several lines of evidence exist supporting the critical role of $1,25(OH)_2D$ and VDR signaling in distal intestinal Ca absorption during growth and it is of vital importance for whole body Ca metabolism and bone health. However, several research questions remain to be answered through future research. For example, although the importance of $1,25(OH)_2D$ and VDR signaling in mediating Ca absorption in the proximal and distal intestine and protecting bone health has been directly tested during growth, its role in adulthood remains unknown. Second, although distal intestine has been shown to be able to actively absorb Ca in a $1,25(OH)_2D$-dependent manner, the translational potential of targeting distal intestine as a therapeutic strategy to attenuate bone loss has not been established. Furthermore, the effect of age on intestinal VDR level and its association with intestinal resistance to $1,25(OH)_2D$ need to be clarified.

This dissertation aims to address these research gaps through three research projects. First, I will systematically test whether $1,25(OH)_2D$/VDR-mediated intestinal Ca absorption is critical for adult bone health. To achieve this goal, we will use adult mice with inducible VDR gene deletion in the entire intestinal epithelium (Villin promoter-$Cre^{ERT2+/-}$ x $VDR^{f/f}$ mice) or in just the distal ileum, cecum, and proximal colon (CDX2-$Cre^{ERT2+/-}$ x $VDR^{f/f}$ mice), and these mice will be fed with either 0.5% (adequate) or 0.2% (low) Ca diet. This will allow me to test three important questions, i.e. “Does VDR deletion in the whole intestine impair intestinal Ca absorption in adult mice, thus disrupting Ca homeostasis and causing bone loss”, “Does VDR deletion in the lower bowel also reduce intestinal Ca absorption in adult mice, which leads to disrupted Ca homeostasis and bone loss?”, and “Will intestinal VDR deletion, either in the whole intestine or just the large intestine, impair the ability to adapt to low dietary Ca and this will increase adult bone loss?”.

Second, I will test if the $1,25(OH)_2D$/VDR-mediated Ca absorptive machinery in the distal intestine can be targeted. Because the traditional oral administration of $1,25(OH)_2D$ does not reach the large intestine due to its rapid absorption and degradation by the small intestine, my experiment will use two novel compounds, a calcitriol glycoside in *Solanum glaucophyllum* (Sg) leaf and a synthetic calcitriol glucuronide, both can be metabolized by bacterial glucuronidases in the large intestine of mice to release free $1,25(OH)_2D$ and specially target the lower bowl. By feeding these compounds to our experimental mice, we will be able to test whether targeting the proximal colon

with glycosylated or glucuronidated forms of calcitriol can stimulate Ca absorption machinery in growing mice. We will also be able to identify the optimal dose of these two compounds that lead to maximal stimulation without causing systemic effect. This work will lay foundations for future research endeavors that test whether these novel compounds can increase Ca absorption in proximal colon and whether this will protect bone mass and microarchitecture in adult mice, especially when dietary Ca is low.

Third, I will thoroughly examine the effect of age on intestinal response to $1,25(OH)_2D$ by measuring duodenal gene expression using RNA-seq analysis across three age groups of mice (i.e., young, mature, old) who were treated with either a single dose of vehicle or $1,25(OH)_2D$ (i.p. 0.5 ng/g BW). This experiment will allow me to answer two questions, i.e. "Is there an age-related decline in duodenal VDR gene expression level?" and "Is the $1,25(OH)_2D$-induced intestinal gene expression suppressed in the aging mice versus the young and mature mice?". The gene oncology and pathway analysis will provide insight into potential gene(s) other than VDR that may contribute to the resistance to $1,25(OH)_2D$ action in aging intestine.

1.9 Figures

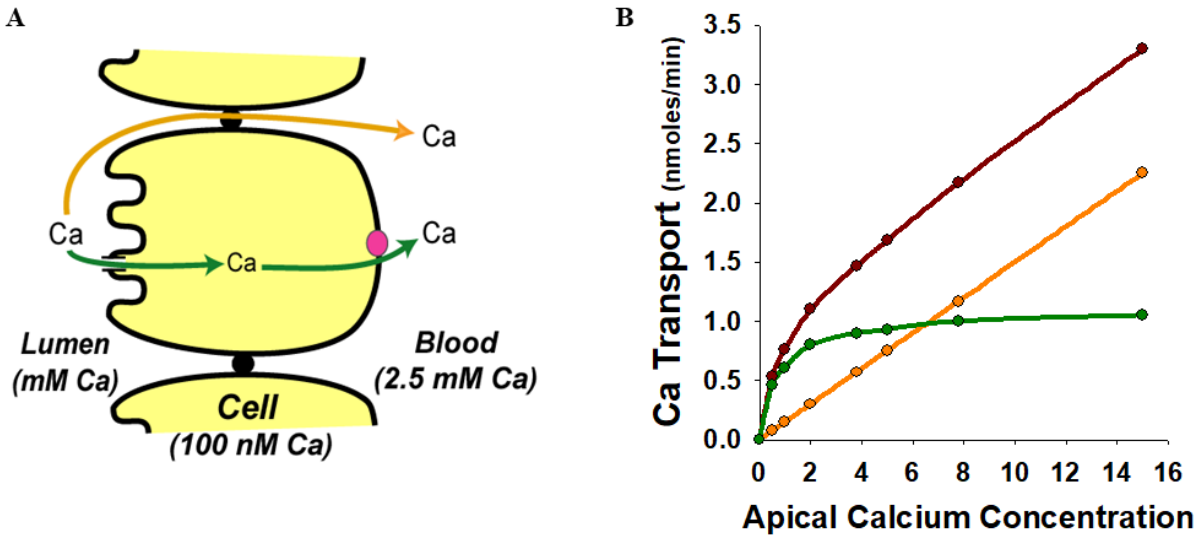

Figure 1.1. Model for Intestinal Calcium Absorption

A. Summary of transcellular and paracellular route for intestinal calcium (Ca) absorption. B. Kinetic modeling of intestinal Ca absorption shows that transcellular route is saturable that can be defined by the Michaelis-Menton equation. In comparison, paracellular is non-saturable and concentration-dependent.

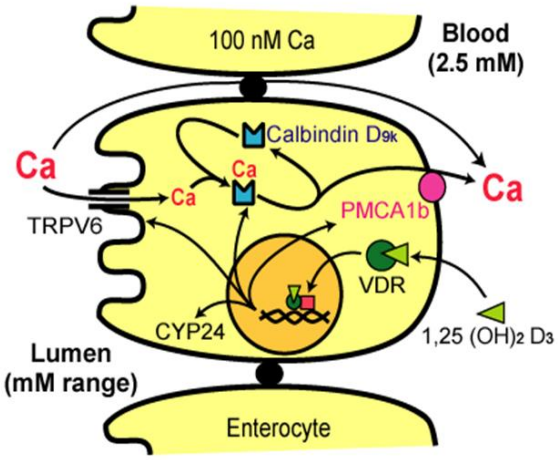

Figure 1.2. Facilitated Diffusion Model of Intestinal Calcium Absorption.

The facilitated diffusion model describes transcellular Ca movement in three steps: Ca transport at the apical membrane via transient receptor potential vanilloid type 6 (TRPV6), Ca across the enterocyte bound to calbindinD9k, and Ca extrusion at the basolateral membrane via a plasma membrane Ca ATPase (PMCA). $1,25(OH)_2D$ is involved in the transcriptional control of each step.

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CHAPTER 2. INTESTINAL VITAMIN D RECEPTOR IN ADULT MICE IS ESSENTIAL TO MAINTAIN BONE MASS WHEN CALCIUM INTAKE IS LOW BUT DISPENSABLE WHEN CALCIUM INTAKE IS ADEQUATE

2.1 Abstract

During rapid bone accrual, $1,25(OH)_2D$/vitamin D receptor (VDR)-mediated intestinal calcium (Ca) absorption is necessary to supply adequate Ca for proper bone mineralization. However, the importance of $1,25(OH)_2D$-mediated intestinal Ca absorption for adult bone maintenance is unknown. We tested the hypothesis that $1,25(OH)_2D$ signaling through the VDR has a significant role in adult intestinal Ca absorption and adult bone health. Transgenic mice were developed to allow inducible VDR gene knockout from the whole intestine (villin promoter-$CreER^{T2+/-}$ x $VDR^{f/f}$ mice, WIK) or from the large intestine (CDX2 promoter-$CreER^{T2+/-}$ x $VDR^{f/f}$ mice, LIK). Littermates with no Cre-ER^{T2} transgene were used as controls. At weaning, mice were fed AIN93G diet (0.5% Ca, 0.4% P, 200 IU VD_3). At 4 month of age, mice received tamoxifen (TAM, 1 mg/20 g body weight, ip for 5 d) to induce recombination of the floxed VDR alleles. After TAM, mice were randomized to AIN93M diet with 0.5% (adequate) or 0.2% (low) Ca. Ca absorption was examined after 2 weeks while serum $1,25(OH)_2D$ and bone mass was examined after 16 weeks on the diets. Intestinal and renal gene expression was measured at both time points (n=12/genotype/diet/time point).

WIK and LIK mice fed adequate Ca had similar levels of renal Cyp27b1 mRNA, serum $1,25(OH)_2D$, Ca absorption, bone mineral content (BMC), and bone mineral density (BMD) as the controls. In control mice, low Ca intake increased renal Cyp27b1 mRNA (+ 3.2-fold at 2-week and + 2.8-fold at 16-week) and serum $1,25(OH)_2D$ level (1.9-fold), which upregulated S100g and Trpv6 gene expression and Ca absorption in the duodenum (+130%) and proximal colon (+30%) (all $p<0.05$). As a result, neither BMC nor BMD was significantly reduced by 16 weeks' low Ca intake in control mice. In WIK mice, the low Ca diet caused a 4.4-fold increase in serum $1,25(OH)_2D$ but the adaptive increases in duodenum and proximal colon Ca absorption and gene expression were lost and BMC and BMD levels were significantly reduced (-28.2 and -23.6%, $p<0.0001$). In contrast, LIK mice adapted to the low Ca diet by increasing serum $1,25(OH)_2D$ (2.5-

fold) that caused adaptive responses in S100g and Trpv6 gene expression and Ca absorption in the duodenum but not proximal colon, resulting in a 12% decline in BMD and BMC ($p<0.05$). Our data suggest that in adult mice, $1,25(OH)_2D$/VDR signaling plays a minimal role in regulating Ca absorption and protecting bone mass when dietary Ca intake is adequate. In contrast, $1,25(OH)_2D$/VDR signaling in the whole intestine, and to a lesser extent the proximal colon, is critical to upregulate Ca absorption and protect bone when dietary Ca intake is low.

2.2 Introduction

Osteoporosis is a global public health concern that brings substantial economic and physical burden to individuals (33). It is characterized by low bone mass and bone structural deterioration, causing bone fragility and increased risk for fracture at multiple sites such as hip, spine, and wrist (32). Increasing PBM and reducing adult bone loss have been considered as key strategies for osteoporosis prevention (118). Intestinal calcium (Ca) absorption has been positively associated with higher PBM during growth (1, 119) and reduced bone loss in adult (44). Higher intestinal Ca absorption can also lower the hip fracture risk (- 2.5-fold) for elderly women with inadequate dietary intake (<400 mg/d) (120).

Given the importance of intestinal Ca absorption for reducing the risk for osteoporosis and fracture, it is critical to understand how it is regulated. The process of intestinal Ca absorption can be presented as the sum of two processes, a saturable and a nonsaturable route (66). The saturable route can be modeled via a Michaelis-Menton equation, it is energy-dependent and is subject to physiological and nutritional regulation. On the contrary, the nonsaturable (presumably paracellular) route is a linear function of the luminal Ca concentration. Under adequate or low Ca intake, total Ca is absorbed predominantly through the saturable Ca absorption route (68). Accumulating evidence have clearly demonstrated that the saturable intestinal Ca absorption is mediated by $1,25(OH)_2D$ signaling. For instance, renal $1,25(OH)_2D$ production and serum $1,25(OH)_2D$ level were elevated with low dietary Ca intake, which then upregulated intestinal Ca absorption efficiency in growing mice and rats (55) (65, 121). The action of $1,25(OH)_2D$ on intestinal Ca absorption is mediated via the vitamin D receptor (VDR) by regulating the expression of genes (i.e., Trpv6, S100g, PMCA1b) that control Ca fluxes through enterocyte (7, 55, 85). When VDR gene is deleted from the whole intestine, Ca absorption decreased by 53% in VDR KO mice. But intestine-specific expression of human VDR transgene in VDR KO mice (KO/TG) restored

intestinal calcium absorption in VDR KO mice. As a result, transgenic intestinal VDR expression prevented the growth arrest, hypocalcemia, hyperthyroidism, and osteomalacia observed in VDR KO mice (8). These findings are direct proof that the regulation of intestinal Ca absorption is the single most important role for $1,25(OH)_2D$ and VDR signaling during growth. The disruption of this signaling leads to substantially reduced Ca absorption and abnormal bone phenotypes during growth.

In addition to the proximal small intestine, accumulating evidence have suggested that the lower bowl also plays an important role to Ca metabolism and bone health. For instance, Ca absorption was significantly lower in patients with Crohn's disease when their colon was removed than in patients with functional colon (12). Similarly, Ca homeostasis was disrupted and bone microstructure was damaged when the large intestine of 8-week-old rats was surgically removed (13, 14). In addition, kinetic studies revealed that $1,25(OH)_2D$ regulates Ca absorption in cecum and colon (9-11). Furthermore, when CDX2 promoter-mediated VDR transgene was expressed to the lower bowl (ileum, cecum, and colon) in 10-12 wks old VDR KO mice, it rescued VDR KO induced abnormalities in Ca metabolism and bone phenotypes such as widened, disorganized growth plate, reduced bone volume and cortical thickness (15). These findings indicate that VDR in the lower bowl contributes substantially to bone health during growth.

Compared to the well-defined role of $1,25(OH)_2D$ and VDR signaling in intestinal Ca absorption and bone health during growth, their significance in adult intestinal Ca absorption and adult bone health has not been formally tested and is under debate. As individuals mature, the need for bone Ca deposition drops substantially as the majority of bone mass has been attained and the bone metabolism is in a balanced state. Intestinal Ca absorption efficiency has also been shown to reduce in adulthood in both human subjects and animal models (16-18, 26, 62). Furthermore, the adaptation to low Ca intake also reduces in adulthood. For instance, a 0.014% very low Ca diet treatment induced a 90% increase among 2-month old young male F344 rats, this adaptation declined to 60% for 4-month old and only 20% for 12-month old rats (23). The reduced ability to adapt to low Ca diet in adult, mature animals has been suggested to be due to reduced renal 1,α -hydroxylase activity which leads to no activation of $1,25(OH)_2D$ signaling (104). These findings have raised the question whether $1,25(OH)_2D$ and VDR signaling is important to intestinal Ca absorption in mature, growth stable adult whose bone mass is stabilized as it is for the rapid growth period. More interestingly, physicians observed that patients with hereditary vitamin D resistant

rickets (HVDRR), who has dysfunctional VDR, can maintain normal serum Ca level and display no bone deformities in their adulthood even without high dose of Ca supplementation(24, 25). Collectively, these evidence suggest that $1,25(OH)_2D$ and VDR may not be important to intestinal Ca absorption and bone health in adult. However, this research question has not been formally tested.

In summary, the literature has showed a significant role of $1,25(OH)_2D$/VDR signaling in intestinal Ca absorption and bone health during growth, and the lower bowl seems to be sufficient in maintaining Ca homeostasis and protect bone during growth. However, we believe there are still a few key questions relating to its role in adult that remain unknown. These questions include: "Is $1,25(OH)_2D$/VDR signaling important to intestinal Ca absorption and bone health in adult with adequate dietary Ca intake?", " Is $1,25(OH)_2D$/VDR signaling important to intestinal Ca absorption and bone health in adult with low dietary Ca intake?", and "Is distal intestine important to adult bone health?". Hence, the goal of our study is to address these questions using adult mice with inducible VDR gene deletion in the entire intestinal epithelium (Villin promoter-$Cre^{ERT2+/-}$ x $VDR^{f/f}$ mice) or in just the distal ileum, cecum, and proximal colon (CDX2-$Cre^{ERT2+/-}$ x $VDR^{f/f}$ mice), and these mice will be fed with either 0.5% (adequate) or 0.2% (low) Ca diet. We hypothesize that a) VDR deletion in the whole intestine will impair intestinal Ca absorption in adult mice, thus disrupting Ca homeostasis and causing bone loss; b) VDR deletion in the lower bowel will also reduce intestinal Ca absorption in adult mice, which leads to disrupted Ca homeostasis and bone loss; c) The impact of VDR deletion in the whole intestine on adult Ca absorption and bone loss will be more severe than that VDR deletion in the lower bowel; d) Intestinal VDR deletion, either in the whole intestine or just the large intestine, will impair the ability to adapt to low dietary Ca and this will increase adult bone loss.

2.3 Material and Methods

2.3.1 Animals

All of the animal experiments were approved by the Purdue Animal Care and Use Committee. Mice were housed in groups of 3-5 mice and exposed to a 12-h light/12-h dark cycle. Food and water were given *ad libitum*.

2.3.2 Generation of mice with whole intestine or large intestine-specific Vdr deletion

Heterozygous mice containing loxP sites flanking exon 4 of the VDR gene ($VDR^{f/w}$) were a gift from Dr. Gardner (University of California San Francisco, CA) (122). Mice with inducible VDR gene deletion in the entire intestinal epithelium (WIK) were made by crossing $VDR^{f/f}$ mice and villin promoter-Cre-ER^{T2} mice (123) to create $VDR^{f/f}$ x Villin-$CreER^{T2+/-}$ mice. Similarly, mice with inducible VDR deletion in distal ileum, cecum, and proximal colon (LIK) were generated by crossing $VDR^{f/f}$ mice with CDX2-Cre-$ER^{T2+/-}$ mice (124) (The Jackson Laborary, Bar Harbor, ME. Stock No: 022390) to create $VDR^{f/f}$ x CDX2-$CreER^{T2+/-}$ mice. Littermates with the VDR floxed alleles but no Cre-ER^{T2} transgene were used as controls. Mouse genotypes were confirmed using genomic DNA isolated from tail clipping with the DNeasy Blood & Tissue Kits (QIAGEN, Hilden, Germany). VDR genotyping was performed using primers described elsewhere (122) with the following PCR conditions: 95 ^{o}C for 2 min, followed by 33 cycles of 95 ^{o}C for 30 sec, 61 ^{o}C for 40 sec, 72 ^{o}C for 1 min 10 sec and a final cycle of 72 ^{o}C for 5 min. VDR genotyping generated a 1.2 kb band for the wild type allele, a 1.5 kb band for the floxed allele, or a 1 kb band for the deletion allele. Cre genotyping of heterozygous mice were carried out using these primers: forward 5'-ACCAGCCAGCTATCAACTCG-3', reverse 5'-TTACATTGGTCCAGCCACC-3'. Additionally, we included a primer pair for the internal control of Cre gene: forward 5'-CTAGGCCACAGAATTGAAAGATCT-3', reverse 5'-GTAGGTGGAAATTCTAGCATCATCC-3'. Amplification of the Cre band started with denaturation at 94 ^{o}C for 3 min, 30 cycles of 94 ^{o}C for 1 min, 60 ^{o}C for 2 min, 72 ^{o}C for 1 min and ended with clean up at 72 ^{o}C for 3 min.

2.3.3 Confirmation of Vdr deletion in WIK and LIK mice

WIK, LIK and control mice (n=8/genotype, balanced by gender) were fed a modified AIN93G diet (0.5% Ca, 0.4% P, 200 IU VD_3) (Research Diets, Inc., New Brunswick, NJ) from weaning. At 4 months of age mice received tamoxifen (1 mg/20 g body weight, i.p. injection for 5 days) to induce recombination of the floxed VDR alleles. 14 d after tamoxifen-treatment, we collected kidney and mucosal scrapings from duodenum, distal ileum, cecum and proximal colon for DNA isolation and VDR genotyping. The primer sequence and PCR conditions for VDR genotyping are the same as described in the previous section.

2.3.4 Experimental design

WIK, LIK and control mice (n=48/genotype, balanced by gender) were fed a modified AIN93G diet (0.5% Ca, 0.4% P, 200 IU VD_3, Research Diets, Inc., New Brunswick, NJ) from weaning. At 4 months of age, mice have reached a plateau for bone Ca accrual and they are considered growth stable, mature adults (22). At this point, mice received tamoxifen (1 mg/20 g body weight, i.p. injection for 5 days) to induce recombination of the floxed VDR alleles. Then mice were randomized to a modified AIN93M diet (0.4% P and 200 IU VD_3) with either 0.5% (adequate) or 0.2% (low) Ca (n=24/genotype/diet). After 2 weeks on the experimental diets, we used 12 mice/genotype/diet (6M, 6F) to assess the short-term effect of diet on: Ca absorption efficiency in the duodenum and the proximal colon using *in situ* ligated loops procedure, gene expression in kidney, duodenum and proximal colon (from the region adjacent to Ca absorption test). After 16 weeks on the experimental diets, we used the remaining 12 mice/genotype/diet to evaluate the long-term effect of diet on serum $1,25(OH)_2D$ level, gene expression in kidney, duodenum and proximal colon, and bone mineral content and density.

2.3.5 Ca absorption test

We assessed the capacity to absorb Ca at duodenum and proximal colon within the same mouse using an *in situ* ligated loops procedure (2 mM Ca, 10 min) as we have previously described (125). The Ca absorption test was done in the proximal colon first by following the procedure below. A small opening was made at about 3 cm distal from the cecocolic junction, through which we removed the fecal remnants in the lumen by gently messaging the loop. Then we tied two ligatures at 0.5 cm and 2.5 cm distal from the cecocolic junction, respectively. ^{45}Ca buffer (transport buffer containing 0.001 mCi/mL ^{45}Ca (Perkin Elmer, Waltham, MA)) was then injected into the lumen of the 2 cm ligated segment of proximal colon. At this concentration of Ca, active Ca transport predominates in the intestine (71, 126). While the proximal colon absorption test was ongoing, the same procedure was repeated at the duodenum (2 cm duodenum segment starting 0.5 cm after the pyloric sphincter). The only exception is that we did not make a cut to remove lumen content because the duodenum was much cleaner than the proximal colon. We took the following precautions to make sure of the quality of our Ca absorption test. First, mice were kept warm and moist throughout the absorption period. Second, we carefully observed and recorded the

appearance and coloring of each loop. Third, we assessed radioactivity leakage of each loop throughout the test with a Geiger counter. At the end of the absorption period, each loop was removed with the loop length measured. Then each segment was collected into a 20 ml glass scintillation vial (Fisher Scientific, Waltham, MA). Afterwards, loops were digested and the radioactivity of ^{45}Ca from the loop was measured as described elsewhere (125). We calculated the efficiency of Ca absorption during the 10-min incubation period by measuring the disappearance of ^{45}Ca from the loop, i.e., [1- (amount of Ca remaining in the loop/amount of Ca injected into the loop)] x 100.

2.3.6 Gene expression

RNA from intestine mucosal scrapings and kidney were isolated using the Zymo Direct-zol RNA kit (Zymo Research, Irvine, CA) according to the manufacturer's directions. The isolated RNA was eluted in 50 µL of water and quantified using a NanoDrop Spectrophotometer 2000 (ThermoFisher Scientific). RNA was then reverse transcribed to cDNA using a reaction cocktail and PCR conditions described elsewhere (125). Afterwards, samples were diluted to 100 ul final volume with autoclaved, distilled deionized water and stored at -20^{o}C. Quantitative real-time PCR (qPCR) was conducted using CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD). Primers (Integrated DNA Technologies, Coralville, IA) and qPCR condition were previously described (125). Fold change of gene expression for Cyp27b1, Trpv6, and S100g were determined using the $2^{-\Delta\Delta CT}$ method normalized relative to Rplp0 described elsewhere (127).

2.3.7 Serum 1,25$(OH)_2$D analysis

Serum 1,25$(OH)_2$D was measured by enzymeimmunoassay following the manufacturer's instructions (Cat No. : AC-62F1. Immuno Diagnostic Systems, Fountain Hills, AZ).

2.3.8 Bone analysis

Upon harvest, the right leg femur was harvested and thoroughly cleaned to remove muscle and connective tissue. Femur samples were then fixed in 10% neutral buffer formalin and stored at 4^{o}C for 7 days. Afterwards, femur samples were rinsed with and stored in 70% ethanol at 4^{o}C until scanning. Scanning was done using a PIXImus densitometer (Lunar; GE-Healthcare,

Madison, WI). Measured variables included bone mineral content (BMC, in grams) and bone mineral density (BMD, in g/cm^2).

2.3.9 Statistical analysis

The statistical analysis was conducted in SAS Enterprise Guide v8.2 (SAS Institute Inc., Cary, NC, USA). All data are presented as mean ± SEM. First, we excluded Ca absorption data based on these criteria: a) intestine loop appeared purple; b) radioactivity leakage. Then we identified additional outliers through the outlier test (z-score < or > two standard deviation). After removing outliers, data was analyzed for normal distribution and for the presence of potential covariates (i.e., body weight, intestine segment length, femur length). Data transformations used to correct non-normality were: square root for duodenum S100g mRNA (2-wk) and serum 1,25$(OH)_2$D level; log10 for proximal colon S100g mRNA (2-wk), duodenum Ca absorption efficiency, kidney Cyp27b1 mRNA (16-wk), and duodenum and proximal colon S100g mRNA (16-wk). Data were analyzed by ANCOVA for the main effects (diet, genotype, gender) and their interactions. When gender did not interact with the other main effects, we removed it from the analysis and conducted a 2-way ANCOVA instead. Differences are considered significant when $p<0.05$.

2.4 Results

2.4.1 Intestine Specific VDR Deletion

We evaluated whether we can successfully delete the VDR gene from the target intestinal regions in WIK and LIK lines. As expected, our TAM treatment (1 mg/20 g body weight, i.p. injection for 5 days) caused complete VDR deletion along the whole intestine in WIK mice. In comparison, LIK mice had limited deletion in distal ileum, complete deletion in cecum and partial deletion in the proximal colon (Fig. 2.1.). Our treatment did not alter the VDR gene in the kidney in either model.

2.4.2 Effect of VDR Deletion on Intestinal Ca Absorption in Adult Mice after 2 Weeks of Dietary Ca Intervention

After 2 weeks of adequate Ca diet, WIK and LIK mice had similar levels of kidney Cyp27b1 gene expression as the Ctrl mice (Fig. 2.2). However, low Ca diet caused a significant increase in kidney Cyp27b1 gene expression for the Ctrl (+ 3.2 fold), WIK (+ 2.2 fold) and LIK (+ 4.6 fold) mice. Similarly, there is no difference in S100g gene expression in the duodenum and proximal colon across different genotypes when mice were fed the adequate Ca diet (Fig. 2.3). But Ctrl mice adapted to the 2 weeks low Ca diet treatment by increasing their S100g gene expression in their duodenum (+ 88%) and proximal colon (+ 9.8-fold). On the contrary, VDR deletion abolished the ability of WIK mice to adapt to low Ca diet because their S100g gene expression is not induced in either the duodenum or the proximal colon. Similar to the Ctrl mice, LIK mice also adapted to the low Ca intake by increasing their duodenal S100g gene expression for 93%, however, they were not able to induce S100g gene expression in the proximal colon due to VDR deletion in this segment. Furthermore, Ca absorption efficiency in the duodenum or proximal colon for the WIK and LIK mice were at similar levels as the Ctrl mice when they were fed adequate Ca diet for 2 weeks (Fig. 2.4). Low Ca diet induced adaptive increase in Ca absorption efficiency in the duodenum (+ 131%) and proximal colon (+ 29%) for the Ctrl mice and in the duodenum only for the LIK mice (+ 66%). In contrast, the WIK mice were not able to adapt to the low Ca diet by increasing intestinal Ca absorption efficiency due to VDR deletion in both the duodenum and proximal colon.

2.4.3 Effect of VDR Deletion on Bone Phenotypes in Adult Mice after 16 Weeks of Dietary Ca Intervention

Similar to the short-term dietary Ca intervention, WIK and LIK mice had similar levels of kidney Cyp27b1 gene expression and serum $1,25(OH)_2D$ level as the Ctrl mice after 16 weeks of adequate Ca diet (Fig. 2.5). However, low Ca diet caused a trend of significant increase in renal Cyp27b1 mRNA (+ 2.8-fold, $p=0.067$) and serum $1,25(OH)_2D$ level (+ 1.9-fold, $p=0.076$) in Ctrl mice. In WIK mice, long-term feeding of the low Ca diet caused a more dramatic increase in renal Cyp27b1 mRNA (+ 12.2-fold, $p<0.0001$) and serum $1,25(OH)_2D$ (+ 4.4-fold, $p<0.0001$) levels, suggesting end organ resistance to $1,25(OH)_2D$ action due to the lack of intestinal VDR in WIK mice. In contrast, LIK mice adapted to the low Ca diet by increasing their kidney Cyp27b1 gene

expression (+ 4.5 fold, p=0.0005) and serum 1,25$(OH)_2$D level (+ 2.5-fold, p=0.005). In addition, the long-term feeding of the adequate Ca diet did not cause any change in S100g (Fig. 2.6) nor Trpv6 (Fig. 2.7.) gene expression in the duodenum and proximal colon across different genotypes. While under low Ca diet, Ctrl mice adapted dietary Ca restriction by increasing their S100g and Trpv6 gene expression in both the duodenum (+ 3.5-fold, + 7.7-fold) and proximal colon (+ 2.6-fold, + 5.5-fold). On the contrary, VDR deletion in the whole intestine of WIK mice eliminated their ability to adapt to the long-term low Ca diet as there is no change in neither gene expression at duodenum and proximal colon. The deletion of VDR from the whole intestine in WIK mice had such a substantial impact that their duodenal Trpv6 level is significantly lower than Ctrl and LIK mice when challenged by the dietary Ca restriction (Fig. 2.7A). Low Ca diet in the LIK mice, however, induced adaptive increase in their duodenal S100g (+ 3.7-fold) and Trpv6 (+ 13.2-fold) gene expression. But there is no induction in gene expression in their proximal colon due to VDR deletion in this segment for LIK mice (Fig. 2.7B). Due to the adaptive responses in both their duodenum and proximal colon for Ctrl mice, the long-term feeding of low Ca diet did not alter their BMD or BMC (Fig. 2.8), indicating physiologic adaptation was sufficient to prevent adult bone loss. In WIK mice, due to the inability to adapt to low Ca diet in both segments, their BMC and BMD were significantly reduced (-28.2% and -23.6%, $p<0.0001$ for both) after the long-term feeding of low Ca diet. Their BMC and BMD level at the end of the intervention were also substantially lower compared to that of the Ctrl and the LIK mice ($p<0.05$). In comparison, the low Ca fed LIK mice had a much milder loss of BMC and BMD (-12% for both).

2.5 Discussion

Our data clearly showed that when adult mice has adequate dietary Ca intake, intestinal VDR is not required for normal Ca metabolism and bone health. Instead, adult mice predominantly rely on VDR-independent Ca absorption for their Ca requirement. This finding of ours is consistent with clinical observations on patients with HVDRR. Normally, in order to attain normal PBM and prevent hypocalcemia, HVDRR patients require treatment of high dose of oral and/or intravenous Ca administration during growth to bypasses the saturable Ca absorption route which depends on functional VDR in the intestine. However, once in their adulthood, these individuals showed remission of the disease with normalization of serum Ca level, comparable level of Ca absorption to age-matched controls, and healing of rickets without the need for high Ca treatment (24, 25,

108, 128, 129). Our findings about the minimal dependence of normal Ca and bone metabolism on intestinal VDR for adult mice is different from what we have known from the growing mice (8, 15, 62). First of all, the basal level of Ca absorption in the duodenum of growing mice (55.4 ± 3.8%, 60-day-old) (62) is much higher than that of the adult mice (14.2 ± 3.08%). Second, VDR deletion from the whole intestine of growing mice resulted in negative outcomes in Ca and bone metabolism. For instance, Song and Fleet (62) demonstrated that when 60-day-old growing mice were fed a diet containing 0.5% Ca, VDR deletion caused a 18-fold increase in serum $1,25(OH)_2D$ level. Duodenal Ca absorption was reduced by 71%, which was associated with significant reduction in S100g mRNA (- 55%), S100g protein (- 47%), and Trpv6 mRNA (- 95%) levels. In contrast, none of these parameters were induced in adult mice when fed adequate Ca in our study, suggesting minimal role of $1,25(OH)_2D$/VDR signaling in adult intestinal Ca absorption and bone health when their Ca need is met.

The huge contrast regarding the dependence of Ca and bone metabolism on intestinal $1,25(OH)_2D$/VDR signaling between adult vs young mice may be attributable to reduced Ca requirement as bone Ca deposition rate reduces significantly in adult after the rapid growth period (19-22). Therefore, adult predominantly rely on VDR-independent Ca transport which is enough for their Ca need under normal physiological conditions. The $1,25(OH)_2D$/VDR signaling-independent Ca absorption occurs through a nonsaturable (presumably paracellular) Ca transport route which was estimated to occur at a rate of 13% per hour of the luminal load (2.2% per 10 min) (130). We expect to see similar rate of Ca absorption in our intestine specific VDR KO mice. However, the duodenal Ca absorption rate (16.85%) for our 4.5-month-old adult WIK is at least 7.7-fold higher than the expected rate. Previous studies on growing VDR KO mice fed adequate Ca diet showed similar levels of duodenal Ca absorption rate as ours (16.3% for 60-day-old) (62). Although the exact mechanism that contribute to VDR-independent intestinal Ca absorption in adult mice remains uncertain, some evidence suggest hormonal regulation of intestinal Ca absorption may be a possible contributor. For example, estrogen has been shown to induce intestinal calcium transport and Trpv6 mRNA levels in VDR KO or vitamin D deficient animals (131-133). Insulin growth factor (IGF-I) was also suggested to enhance duodenal Ca absorption in 2-mo-old VDR KO mice which leads to the normalization of plasma Ca and osteomalacia (62). Therefore, it's possible that Ca absorption shifts from predominantly $1,25(OH)_2D$/VDR-dependent mechanism in growing individuals to primarily non $1,25(OH)_2D$/VDR-dependent mechanism that

involves other hormones such as estrogen or IGF-I. However, future research are needed to identify the exact mechanisms.

A second key contribution of our study to the literature is that we extended the examination of 1,25$(OH)_2$D/VDR signaling in adult mice by including the low Ca diet treatment. We found that opposite to the adequate Ca diet, dietary Ca restriction stimulated the dependence of intestinal Ca absorption and bone health on intestinal VDR. Renal 1,25$(OH)_2$D synthesis and serum 1,25$(OH)_2$D level were elevated in Ctrl mice, which lead to an increase in intestinal Trpv6 and S100g gene expression, and Ca absorption in both duodenum (+131%) and proximal colon (+28.8%). In contrast, the loss of functional VDR in the whole intestine eliminated 1,25$(OH)_2$D/VDR-mediated intestinal adaptive responses. Consequently, the long-term feeding of low Ca diet to mice with intestinal VDR deletion caused bone loss as there is a 28% reduction in BMC and a 24% reduction in BMD for the WIK mice. These findings suggest that intestinal 1,25$(OH)_2$D/VDR signaling plays a significant role in adult intestinal Ca absorption and bone health under dietary Ca restriction. Our findings is opposite to previous studies that showed no activation of intestinal 1,25$(OH)_2$D signaling due to reduced renal 1α-hydroxylase activity in 4-month-old male rats (104). The extent of elevation in duodenal Ca absorption in response to low Ca intake in our 4.5-month-old adult mice is also higher than previously reported (40% increase in 4-month-old rats) (23). Due to the normal 1,25$(OH)_2$D/VDR-mediated adaptations to low Ca diet, our adult mice were able to maintain their BMC and BMD.

A third key finding of our study is that proximal colon in adult mice has Ca absorptive capacity and it is important for 1,25$(OH)_2$D-mediated adaptive responses to low Ca intake. The basal Ca absorption efficiency in the proximal colon of our adult mice is 23.97 ± 2.47% for the Ctrl. It is similar to the level in mice of other genotypes: 25.42 ± 2.68% for the WIK, and 25.38 ± 3.02%, suggesting VDR-independent basal Ca absorption in the proximal colon. However, VDR is needed for enhancing Ca absorption under low Ca dietary intake and the deletion of VDR in the proximal colon of WIK and LIK mice abolished 1,25$(OH)_2$D/VDR signaling-mediated adaptation to the low Ca intake. In comparison, low Ca diet in Ctrl mice induced adaptive responses in the proximal colon such as increased gene expression and Ca absorption. Our findings are consistent with previous studies that suggested the machinery for 1,25$(OH)_2$D-mediated intestinal Ca absorption exists in the lower bowel of growing animals (10, 11, 97, 134). Our study, however, is the first to directly measure and compare Ca absorption capability between proximal colon and

duodenum of adult mice *in vivo*. We found that proximal colon demonstrated significant but much lower responsiveness to 1,25$(OH)_2$D signaling as the duodenum to enhance the Ca absorption capacity under dietary Ca restriction: Ca absorption efficiency increased from 23.97 ± 2.47% to 30.89 ± 2.14% (+ 29%) in the proximal colon of the Ctrl mice versus from 14.20 ± 3.08% to 32.83 ± 5.26% (+ 131%) in their duodenum. These results suggest that adult mice predominantly rely on the proximal intestine for enhancing Ca absorption under dietary Ca restriction. Although previous study indicate that for growing mice, proximal intestine plays a minimal role in Ca metabolism (96). Instead, the lower bowl might be the single most important portion of the intestine for Ca and bone metabolism during growth when VDR transgene is expressed only in the lower bowl (15). We evaluated the relative importance of the lower bowl in adult bone health under the setting of the whole body system. Our finding is different from what has been discovered for growth. When VDR is deleted from the lower bowl, LIK mice showed a -12% reduction of femoral BMC and BMD. Although significant, it is much less than the decline of BMC (-28.2%) and BMD (-23.6%) in WIK mice due to VDR deletion from the whole intestine epithelium. Our results suggest that when VDR is functional in the proximal small intestine, the lower bowl plays a less significant role in maintaining bone mass for adult mice.

In summary, our study reveals important facets of the role of 1,25$(OH)_2$D and VDR signaling in adult Ca absorption and adult bone health. First, we clearly show that 1,25$(OH)_2$D and VDR play a minimal role in Ca absorption and bone health in adult as long as their dietary Ca intake is adequate. This directly relates to HVDRR patients in their adulthood: it is particularly important for these individuals to have adequate daily Ca intake in order to maintain their bone mass. Our findings also reveal that VDR-independent Ca absorption is dominant in adult with adequate Ca intake, the mechanisms of which need further investigations. Second, our work about VDR in adult extends what are known of VDR role in growing individuals and provides additional evidence against the doubt over the role of 1,25$(OH)_2$D and VDR in intestinal Ca absorption and bone health (135). Our findings indicate they are critical for inducing adaptive responses in Ca absorption and protecting against adult bone loss when Ca intake is low. Third, we demonstrated for the first time in vivo that adult proximal colon is capable to actively absorb Ca with the induction of 1,25$(OH)_2$D under low Ca diet. Our previous work found that 1,25$(OH)_2$D-glycosides in Solanum Glaucophyllum leaf can release active 1,25$(OH)_2$D and stimulate Ca absorptive machinery in the proximal colon of growing 3-mo-old mice (125). Therefore, our current work

lays the foundation for future research that tests the effectiveness of this novel compound on promoting Ca absorption in the lower bowl of adult animals. If successful, targeting proximal colon with this compound can be a safe way to improve Ca homeostasis and attenuate bone loss in adult at high risk for bone loss due to conditions such as bariatric surgery, short bowel syndrome (136) (137) (138) (139).

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2.7 Figures and Tables

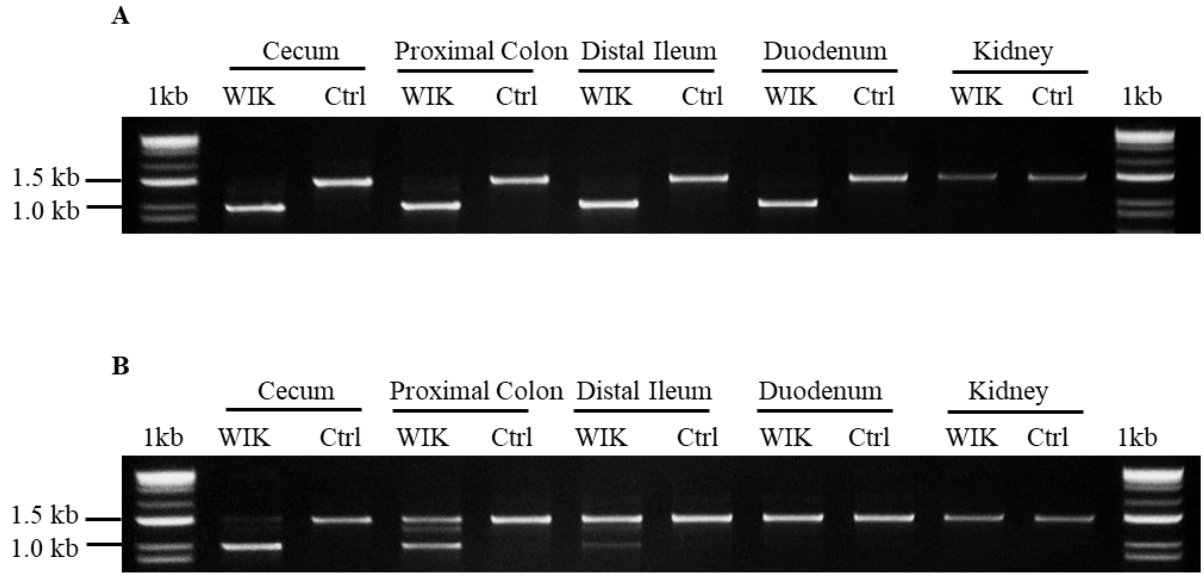

Figure 2.1.Generation of Intestine Specific VDR Deletion Mice

Four-month old whole intestine VDR knockout (WIK) (A) and large intestine VDR knockout (LIK) (B) mice received tamoxifen-treatment (1 mg/20 g BW, ip, 5 days). Mucosal scrapings from cecum, proximal colon, distal ileum, and duodenum and kidney were collected 14 d after for DNA extraction/VDR genotyping. Genotyping yields a 1.5 kb band for VDR floxed allele, a 1.2 kb for the wild type allele, a 1.0 kb band for the deletion allele.

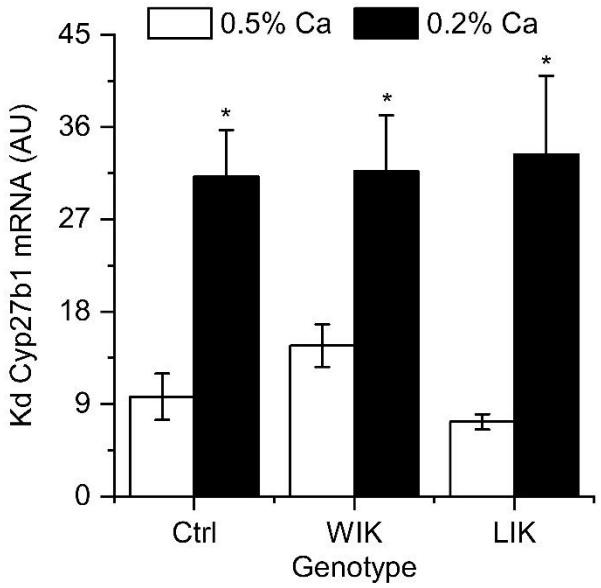

Figure 2.2. Renal Cyp27b1 Gene Expression in Response to Short-term Low Dietary Ca Intake.

Four-month-old control (Ctrl), whole intestine VDR knockout (WIK), and large intestine VDR knockout (LIK) mice received tamoxifen (1 mg/20g BW, i.p., 5 days) to induce recombination of the floxed VDR alleles. Afterwards, mice were randomized to AIN93M diet with either 0.5% or 0.2% Ca. After two weeks, RNA was isolated from kidney (Kd) and examined for Cyp27b1 mRNA by real-time PCR. AU: arbitrary units. Data are expressed as mean ± SEM (n=8-11 per group). *$p<0.05$ vs 0.5% Ca group of the same genotype.

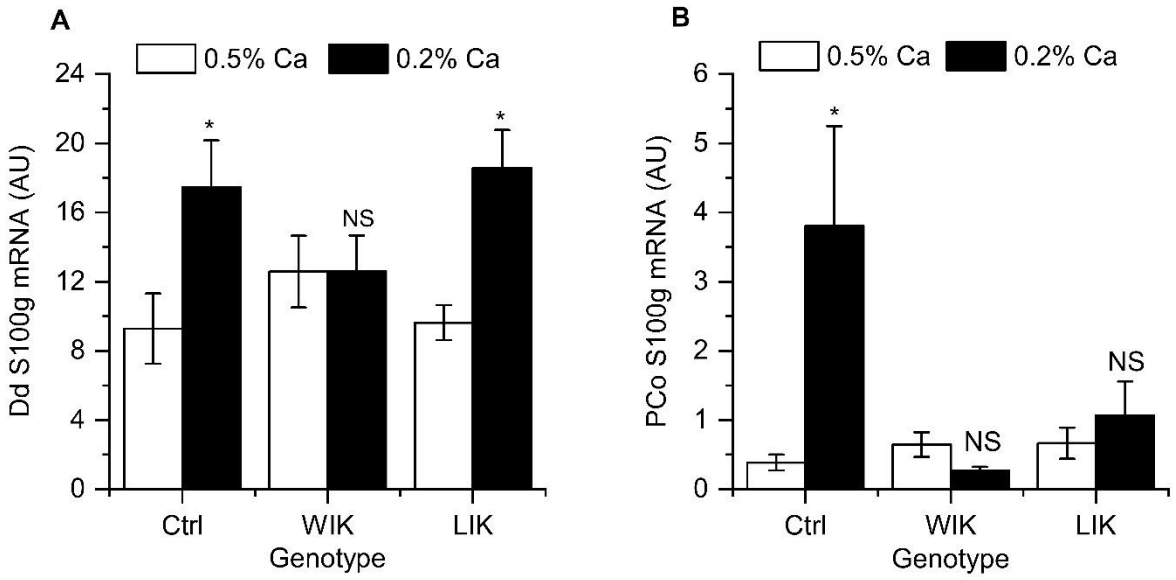

Figure 2.3. Intestinal S100g Gene Expression in Response to Short-term Low Dietary Ca Intake.

Four-month-old control (Ctrl), whole intestine VDR knockout (WIK), and large intestine VDR knockout (LIK) mice received tamoxifen (1 mg/20g BW, i.p., 5 days) to induce recombination of the floxed VDR alleles. Afterwards, mice were randomized to AIN93M diet with either 0.5% or 0.2% Ca. After two weeks, RNA was isolated from the (A) duodenum (Dd) and (B) proximal colon (PCo) and examined for S100g mRNA by real-time PCR. AU: arbitrary units. Data are expressed as mean ± SEM (n=8-11 per group). *$p<0.05$ vs 0.5% Ca group of the same genotype. NS: non-significant.

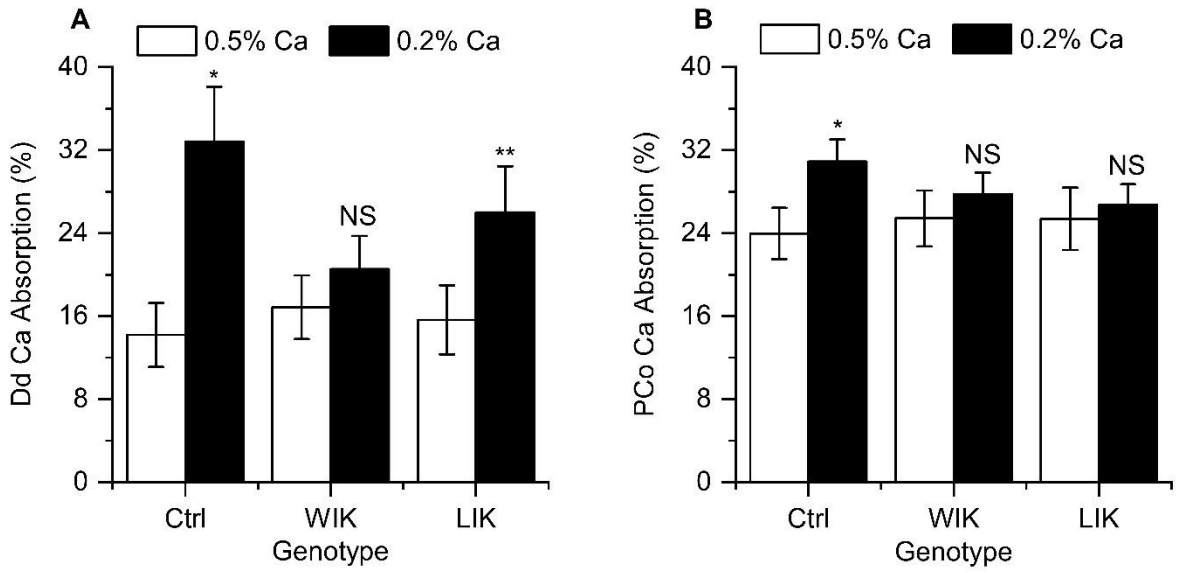

Figure 2.4. Intestinal Ca Absorption Efficiency in Response to Short-term Low Dietary Ca Intake.

Four-month-old control (Ctrl), whole intestine VDR knockout (WIK), and large intestine VDR knockout (LIK) mice received tamoxifen (1 mg/20g BW, i.p., 5 days) to induce recombination of the floxed VDR alleles. Afterwards, mice were randomized to AIN93M diet with either 0.5% or 0.2% Ca. After two weeks, we simultaneously assessed Ca absorption efficiency (%) at duodenum (Dd) and proximal colon (PCo) of the same mouse using the *in situ* ligated loops (2 cm segments, 2 mM Ca, 10 min). Data are expressed as mean ± SEM (n=9-11 per group), *$p<0.05$ vs 0.5% Ca group of the same genotype. **$p=0.083$ vs 0.5% Ca group of the same genotype. NS: non-significant.

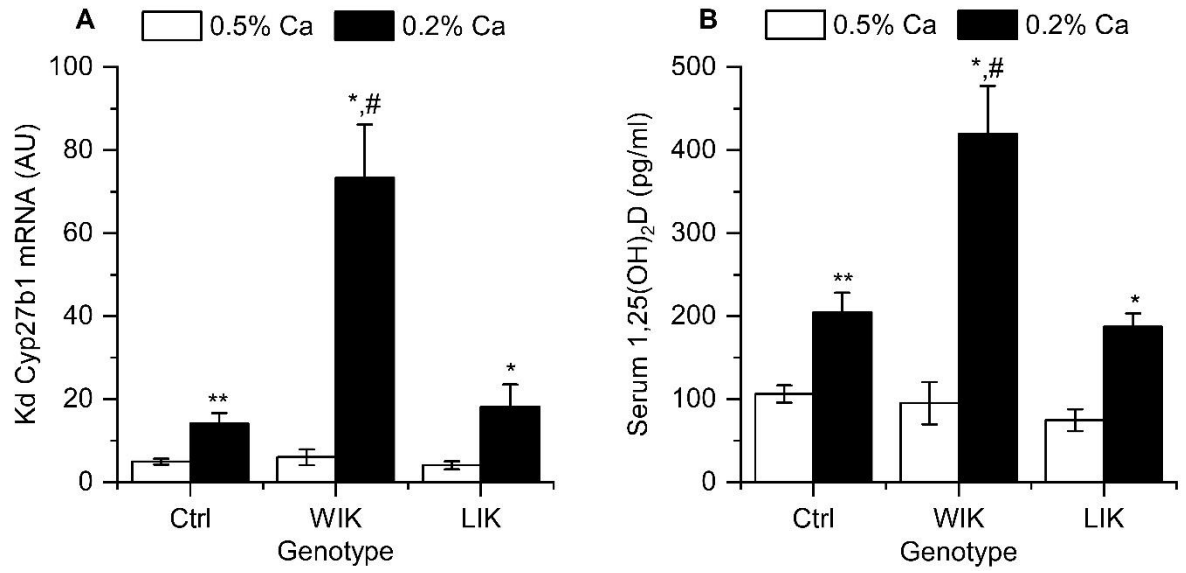

Figure 2.5. Renal Cyp27b1 Gene Expression and Serum $1,25(OH)_2D_3$ Level in Response to Long-term Low Dietary Ca Intake.

Four-month-old control (Ctrl), whole intestine VDR knockout (WIK), and large intestine VDR knockout (LIK) mice received tamoxifen (1 mg/20g BW, i.p., 5 days) to induce recombination of the floxed VDR alleles. Afterwards, mice were randomized to AIN93M diet with either 0.5% or 0.2% Ca. After sixteen weeks, we assessed renal Cyp27b1 mRNA (A) by real-time PCR and serum $1,25(OH)_2D_3$ level (B) by RIA. AU: arbitrary units. Data are expressed as mean ± SEM (n=8-11 per group). *$p<0.05$ vs 0.5% Ca group of the same genotype. **$p<0.1$ vs 0.5% Ca group of the same genotype. #$p<0.05$ vs all other groups.

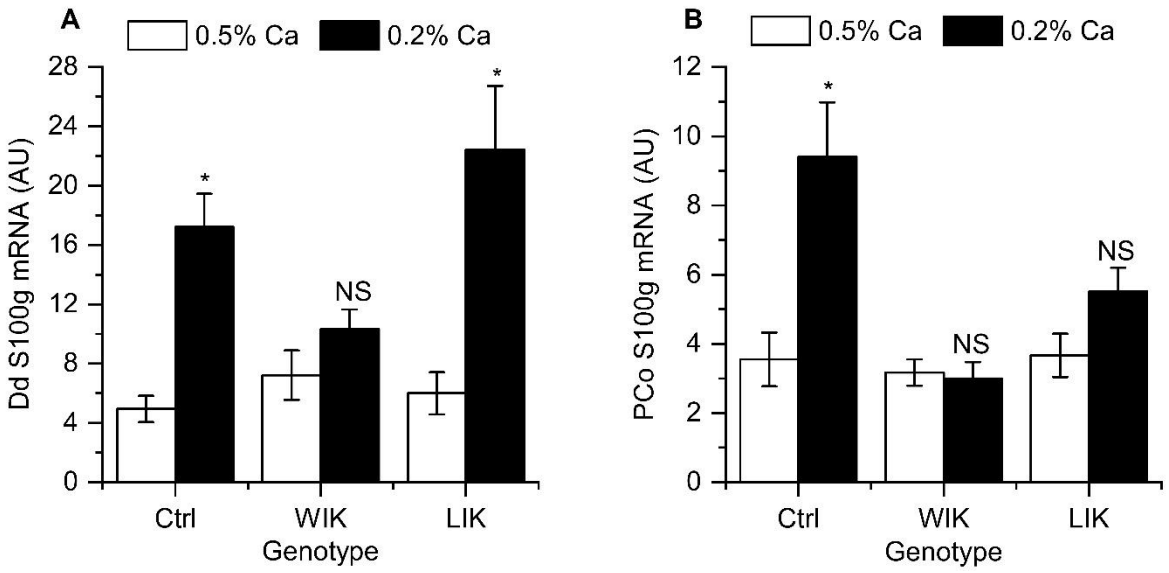

Figure 2.6. Intestinal S100g Gene Expression in Response to Long-term Low Dietary Ca Intake.

Four-month-old control (Ctrl), whole intestine VDR knockout (WIK), and large intestine VDR knockout (LIK) mice received tamoxifen (1 mg/20g BW, i.p., 5 days) to induce recombination of the floxed VDR alleles. Afterwards, mice were randomized to AIN93M diet with either 0.5% or 0.2% Ca. After sixteen weeks, RNA was isolated from the (A) duodenum (Dd) and (B) proximal colon (PCo) and examined for S100g mRNA by real-time PCR. AU: arbitrary units. Data are expressed as mean ± SEM (n=8-11 per group). *$p<0.05$ vs 0.5% Ca group of the same genotype. NS: non-significant.

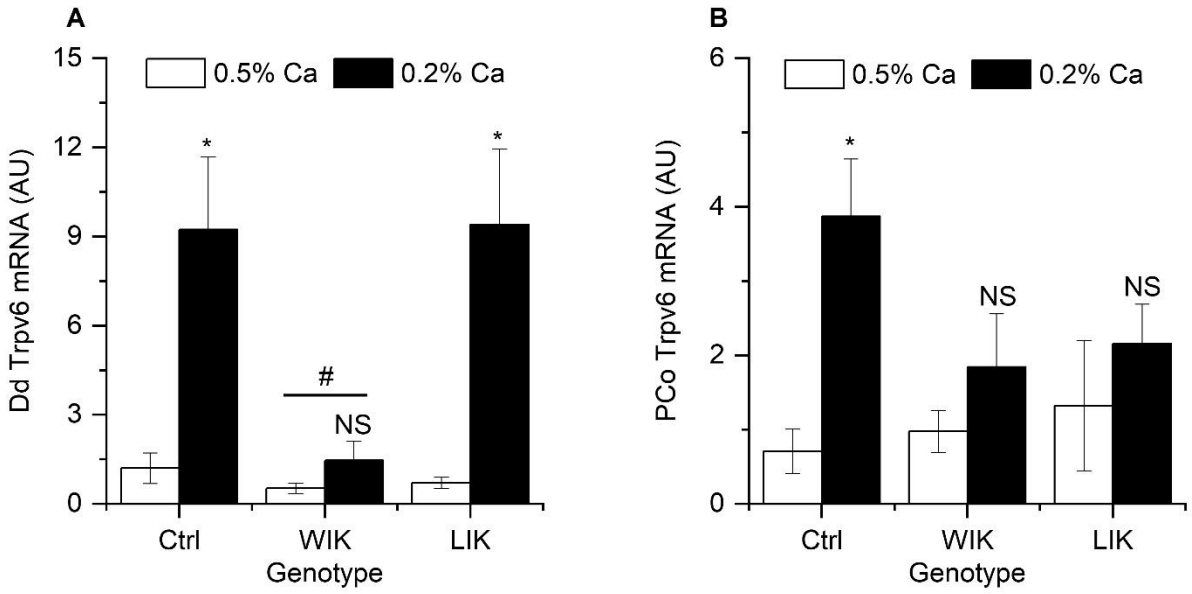

Figure 2.7. Intestinal Trpv6 Gene Expression in Response to Long-term Low Dietary Ca Intake.

Four-month-old control (Ctrl), whole intestine VDR knockout (WIK), and large intestine VDR knockout (LIK) mice received tamoxifen (1 mg/20g BW, i.p., 5 days) to induce recombination of the floxed VDR alleles. Afterwards, mice were randomized to AIN93M diet with either 0.5% or 0.2% Ca. After sixteen weeks, RNA was isolated from the (A) duodenum (Dd) and (B) proximal colon (PCo) and examined for Trpv6 mRNA by real-time PCR. AU: arbitrary units. Data are expressed as mean ± SEM (n=8-11 per group). *$p<0.05$ vs 0.5% Ca group of the same genotype. #$p<0.05$ vs 0.2% Ca fed Ctrl and LIK mice. NS: non-significant.

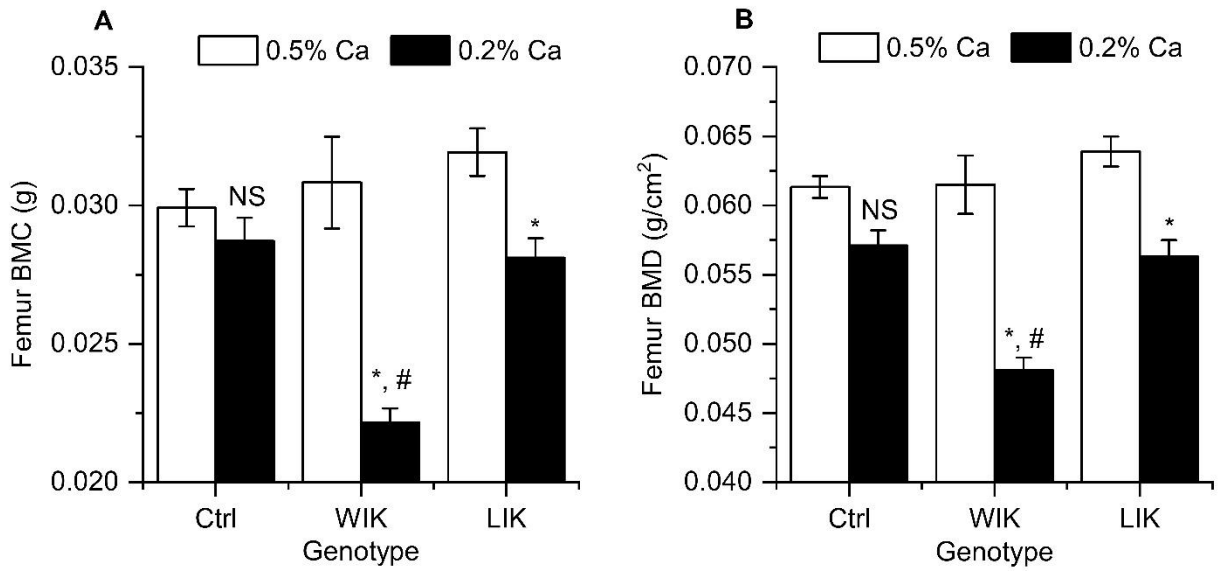

Figure 2.8. Bone Mineral Content and Density in Response to Long-term Low Dietary Ca Intake.

Four-month-old control (Ctrl), whole intestine VDR knockout (WIK), and large intestine VDR knockout (LIK) mice received tamoxifen (1 mg/20g BW, i.p., 5 days) to induce recombination of the floxed VDR alleles. Afterwards, mice were randomized to AIN93M diet with either 0.5% or 0.2% Ca. After sixteen weeks, the right leg femur was scanned using a PIXImus densitometer for bone mineral content (BMC) and density (BMD). Data are expressed as mean ± SEM (n=12 per group). *$p<0.05$ vs 0.5% Ca group of the same genotype. #$p<0.05$ vs all other groups. NS: non-significant.

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CHAPTER 3. TARGETING 1,25(OH)$_2$D-MEDIATED CALCIUM ABSORPTION MACHINERY IN PROXIMAL COLON WITH CALCITRIOL GLYCOSIDES AND GLUCURONIDES

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3.1 Abstract

High intestinal calcium (Ca) absorption efficiency is associated with high peak bone mass in adolescents and reduced bone loss in adulthood. Transepithelial intestinal Ca absorption is mediated by 1,25-dihydroxyvitamin D (1,25(OH)$_2$D, calcitriol) through the vitamin D receptor (VDR). Most research on Ca absorption focuses on the proximal small intestine but evidence shows that large intestine plays a crucial role in whole body Ca homeostasis. We directly assessed and compared Ca absorption capacity at the proximal colon and duodenum using *in situ* ligated loops (2 mM Ca, 10 min). In C57BL/6J mice, the proximal colon (26.2 ± 3.7%) had comparable ability to absorb Ca as the duodenum (30.0 ± 6.7%). In VDR knockout (KO) mice, Ca absorption efficiency was reduced by 67% in duodenum and 48% in proximal colon. These data suggest that large intestine could be targeted to improve Ca absorption and protect bone in at risk-groups (e.g. bariatric patients). Glycoside forms of calcitriol found in *Solanum Glaucophyllum* (Sg) leaf are biologically inert but can be activated in the colon upon bacterial cleavage of the glycosides. We conducted a study to test whether Sg leaf, as well as a novel, synthetic 1,3-diglucuronide form of calcitriol (1,3-diG) could target the proximal colon and upregulate genes involved in Ca absorption (i.e. Trpv6, S100g). 13-week-old female C57BL6/J mice were fed AIN93G diet containing increasing levels of one of the two compounds for 2 weeks (delivering 0, 0.25, 0.5, 1, or 2 ng calcitriol equivalent per day). Both compounds induced a dose-dependent upregulation of Cyp24a1 and Trpv6 gene expression in the proximal colon. 1,3-diG also induced S100g gene expression in the proximal colon. Duodenal expression of Trpv6 was upregulated at higher doses of 1,3-diG but not Sg leaf. These data suggest that both glycosylated and glucuronidated calcitriol could be used to target the proximal colon but that dosing must be optimized to limit systemic

effects that could cause hypercalcemia. Future studies will test the translational potential of these compounds to determine if they can increase Ca absorption at proximal colon and whether this can help protect bone.

Key Words: Solanum Glaucophyllum, 1,25-dihydroxyvitamin D, calcium, colon

Abbreviations:
$1,25(OH)_2D$ = 1,25-dihydroxyvitamin D
Ca = calcium
Cyp24a1 = cytochrome p450, Family 24, subfamily A, member 1 (24 hydroxylase)
PMCA1b = plasma membrane calcium ATPase isoform 1b
S100g = calcium binding protein or calbindin D_{9k}
Sg = *Solanum Glaucophyllum*
Trpv6 = transient receptor potential cation channel subfamily V member 6
VDR = Vitamin D receptor

3.2 Introduction

Intestinal calcium (Ca) absorption brings Ca from ingested food into the body. It is part of a three-tissue axis, including bone and kidney that regulates whole body Ca homeostasis. High Ca absorption efficiency has been linked to high peak bone mass in adolescents (1, 119) and reduced bone loss in adulthood (4, 140). It is also positively correlated to femur BMD, trabecular bone volume (BV/TV), and trabecular thickness in a genetically diverse population of growing mice (2).

Kinetics analysis across a wide range of luminal Ca concentrations demonstrated that intestinal Ca absorption follows both a saturable and a non-saturable pathway (71). The relationship between these two transport pathways can be modeled mathematically by a Michaelis-Menton-like equation that is modified to include a linear component that reflects passive diffusion through tight junctions. Saturable Ca transport pathway is prevalent in the proximal small intestine and is regulated by nutritional and physiological conditions. Under habitual low to adequate dietary Ca intake, saturable Ca transport will account for >60% of total Ca absorption in the small intestine (141). In contrast, non-saturable Ca transport occurs at a constant rate across the intestine lumen (13% of luminal load per hour) (68, 71).

Intestinal Ca absorption was first reported to be dependent on vitamin D in 1937 (142) and other research shows that intestinal Ca absorption efficiency was reduced by 75% in vitamin D deficient rats (66). The bulk of the evidence suggests that the effect of vitamin D on Ca absorption is limited to the saturable Ca component and this is due to the induction of three proteins: the apical membrane Ca channel Trpv6, the intracellular Ca binding protein calbindin D_{9k} (encoded by the S100g gene), and the basolateral membrane Ca ATPase PMCA1b (141). The genes for each of these proteins are transcriptionally regulated by 1,25-dihydroxyvitamin D ($1,25(OH)_2D$)-mediated activation of the vitamin D receptor (VDR) (7, 55, 85). Previously, we showed that expression of a human VDR transgene throughout the intestinal epithelium could restore intestinal Ca absorption and prevent the growth arrest, hypocalcemia, hyperthyroidism, and osteomalacia observed in VDR KO mice (8). This illustrates the critical role that intestinal VDR and vitamin D-mediated Ca absorption play in the control of whole body Ca metabolism during growth.

While mechanistic studies on Ca absorption have focused on the proximal small intestine, several studies suggest that Ca absorption in the large intestine could be important for whole body Ca metabolism. For instance, Ca absorption was significantly higher for patients with Crohn's disease when their colon is not removed (12). In addition, studies in rodents showed that active Ca absorption exists in the colon and cecum (9, 10) and that $1,25(OH)_2D$ injection significantly increased absorptive Ca flux and net Ca transport in colon (11). All these studies indicate that the machinery for vitamin D-mediated intestinal Ca absorption exists in the lower bowel. Furthermore, research by Dhawan et al. (15) has shown that vitamin D-mediated events in the lower bowel are a critical contributor to whole Ca metabolism and bone health. They found that transgenic VDR expression directed to the distal ileum, cecum, and colon was sufficient to prevent the abnormal Ca metabolism normally seen in VDR KO mice. Collectively, these studies suggest the distal intestine could be targeted with $1,25(OH)_2D$ to enhance Ca absorption and bone health. This could be useful for individuals at risk for bone loss due to conditions such as bariatric surgery, short-bowel syndrome, or aging.

Orally administered $1,25(OH)_2D$ does not reach the large intestine because it is rapidly absorbed by the small intestine before it can reach the colon. This also leads to significantly elevated plasma concentrations of the hormone (143). Therefore, a novel strategy is needed to deliver the hormone specifically to the colon. *Solanum glaucophyllum* (Sg) is a plant that grows in South America whose consumption causes high blood Ca level, soft tissue calcification, and

wasting in grazing ruminant animals (144). This is due to the presence of $1,25(OH)_2D$-glycosides in Sg leaf (145) (146). The glycoside forms of $1,25(OH)_2D$ in Sg are biologically inert until the glycosides are cleaved by bacteria with β-glycosidase activity that reside in the rumen (147). This capacity also exists in the lower intestine of monogastric animals and account for why rodent studies have identified doses of Sg leaf that induce expression of the vitamin D-dependent gene, Cyp24a1, specifically in the colon without causing systemic effects (147). Similar, colon-targeted effects have also been observed with synthetic, glucuronidated forms of $1,25(OH)_2D$ (143, 148).

Here, our goal is to investigate whether the proximal colon has vitamin D-regulated Ca absorption that is dependent upon the presence of the VDR. In addition, we tested whether doses of Sg leaf, as well as a novel, synthetic 1,3-diglucuronide form of calcitriol, could be optimized to upregulate genes controlling intestinal Ca absorption in the proximal colon of mice without having systemic effects.

3.3 Material and Methods

3.3.1 Dietary preparation

Sg leaf powder, 25-hydroxyvitamin D_3-25-β-glucuronide-25(OH)D (β-gluc-25(OH)D), and the 1,3-diglucuronide-$1,25(OH)_2D$ were provided by GlycoMyr, Inc. (Ames, IA). Powdered, modified AIN93G diet (0.5% Ca, 0.4% P and 0.2 IU/g vitamin D_3) (Research Diet Inc., New Brunswick, NJ) was mixed with 25-hydroxyvitamin D_3-25-β-glucuronide-25(OH)D (β-gluc-25(OH)D) (0.247 nmol/g diet) to make a diet base. Previous research has shown that the addition of β-gluc-25(OH)D to a diet containing β-25-monoglucuronide-$1,25(OH)_2D$ (βGluc-$1,25(OH)_2D$) enhanced the effectiveness of the compound as a treatment for DSS-induced colitis in mice (143). Sg leaf or 1,3-diglucuronide-$1,25(OH)_2D$ were then mixed with the base diet to deliver a dose equivalent to 0.07, 0.14, 0.28, or 0.57 ng calcitriol per gram diet. Assuming an average consumption of 3.5 g of diet per day per mouse, these concentrations deliver 0.25, 0.5, 1, or 2 ng calcitriol equivalents per day per mouse. In order to deliver the desired amount of calcitriol equivalents, Sg leaf (10 ng calcitriol activity/mg leaf) was mixed in the diet at a level of 7.14, 14.28, 28.56, or 57.12 µg/g diet while we added 1,3-diglucuronide-$1,25(OH)_2D$ (54% calcitriol) at 0.13, 0.26, 0.52, or 1.04 ng/g diet. After mixing, diet was pelleted using a model ECO-3 pellet mill (Colorado Mill Equipment, Canon City, CO). Pelleted diet was stored at 4°C until use.

3.3.2 Animals

All mice were on the C57BL/6J background. C57BL/6J mice and mice with deletion of exon 3 in the VDR gene (VDR KO) (Stock number 006133) were obtained from the Jackson Laboratories. VDR KO mice were maintained as a breeding colony of heterozygous mice at Purdue University. Mice were housed in groups of 3-5 mice and exposed to a 12-h light/12-h dark cycle. Food and water were given *ad libitum*.

3.3.3 Experimental design

Experiment I: Weanling VDR KO mice were raised on rescue diet (AIN93G base modified to have 20% lactose, 2% Ca, 1.25% P, 2200 IU/kg vitamin D_3) and wild type (WT) littermates were fed AIN93G diet (0.5% Ca, 0.4% P, 1000 IU/kg vitamin D_3) (Research Diets, Inc., New Brunswick, NJ) (n=8-10 per genotype). When mice were 7-10 weeks old, the capacity to absorb Ca at duodenum and proximal colon was assessed simultaneously within the same mouse using a modification of the *in situ* ligated loops procedure we have previously described (62). To minimize any potential impact of diet remaining in the lumen, mice were fasted overnight and loops were flushed with a transport buffer (40 µL; 2 mM $CaCl_2$, 150 mmol/L NaCl, 30 mmol/L Tris) prior to the absorption test. ^{45}Ca buffer (transport buffer containing 0.001 mCi/mL ^{45}Ca (Perkin Elmer, Waltham, MA)) was then injected into the lumen of a 2 cm segment of the proximal colon. At this concentration of Ca, active Ca transport predominates in the small intestine (71, 126). While the proximal colon absorption test was ongoing, the same procedure was repeated at the duodenum. For each segment, Ca absorption was allowed to proceed for 10 min, after which time, the loop was removed and digested in 3 mL of SOLVABLE (Perkin Elmer, Waltham, MA) at 60°C for 4-6 hours. H_2O_2 was mixed with the digested mixture (300 µL per mL of digested tissue) to correct for color quenching. Afterwards, a 100 µL aliquot of the mixture was added to 12 mL Ultima Gold (Perkin Elmer, Waltham, MA) and the radioactivity was read on a liquid scintillation counter (Perkin Elmer Tri-Carb 2910TR). The efficiency of Ca absorption during the 10-min incubation period was determined by measuring the disappearance of ^{45}Ca from the loop, i.e., [1- (amount of Ca remaining in the loop/amount of Ca injected into the loop)] x 100.

Experiment II: Female C57BL/6J mice were fed a modified AIN93G diet (0.5% Ca, 0.4% P and 200 IU/kg vitamin D_3, Research Diet Inc.) from 4-13 weeks of age. Mice (n=5 per group) were then randomized to either the β-gluc-25(OH)D-containing base diet (0 ng calcitriol) or to diets that contained 0.25, 0.5, 1, or 2 ng calcitriol equivalent per day as either Sg leaf or 1,3-diglucuronide-1,25$(OH)_2$D. After a two-week feeding period, mice were euthanized and intestine mucosal scrapings were harvested from duodenum, distal ileum, cecum, and proximal colon. Mucosal scrapings were collected from specific intestine segments as follows: duodenum was the 2 cm segment starting 0.5 cm after the pyloric sphincter; ileum was the 4 cm segment starting 0.5 cm proximal to the ileocecal valve; proximal colon was the 2 cm segment starting 0.5 cm distal from the cecocolic junction. Intestinal scrapings were placed in TriReagent (Zymo Research, Irvine, CA) then immediately frozen at -80ºC until analysis.

3.3.4 Tissue and RNA processing

RNA from intestine was isolated using Direct-zol RNA Miniprep Kit (Zymo Research, Irvine, CA) according to the manufacturer's directions. The isolated RNA was eluted in 50 µL of water and quantified using a NanoDrop Spectrophotometer 2000 (ThermoFisher Scientific). 0.5 µg RNA was then mixed with reaction cocktail containing the following reagents (Invitrogen, Carlsbad, CA): 5x first strand buffer, 0.05 mg/ml BSA, 0.05 mM each of dATP, dTTP, dGTP, and dCTP, 0.6 U/ul Rnasin, 5 ng/ul random hexamers, 5 ng/ul oligo-dT, and 10 U/ul M-MLV. RNA was then reverse transcribed to cDNA in a 10 µL reaction volume under the following conditions: 37 ºC for 1 hour, 95 ºC for 10 min, 4 ºC to cool. Afterwards, samples were diluted to a 100 ul final volume with sterile, distilled deionized water and stored at -20 ºC prior to PCR analysis.

3.3.5 Gene expression

Quantitative real-time PCR (qPCR) was conducted with the CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD) using FAM-labeled primers from Integrated DNA Technologies (Coralville, IA): mRplp0 (Mm.PT.56a.42153953), forward 5'-CTGAGTACACCTTCCCACTT-3', reverse 5'-CTCTTCCTTTGCTTCAGCTTTG-3', probe 5'-AGATGGATCAGCCAGGAAGGCC-3'; mTrpv6 (Mm.PT.58.9749660), forward 5'-CATGCTTAACCTCCTCATTGC-3', reverse 5'-TTCCGCTCTAACATCACAGTG-3', probe 5'-

TCATCTCGCTCATGGGCAACTCTC-3'; mCyp24a1 (Mm.PT.58.30780707), forward 5'-AAGAACTGTACGCTGCTGTC-3', reverse 5'-GGGATTCCGGGATAGATTGTAG-3', probe 5'-TCAAGCTGTTTGCGGTCGTCTCC-3'; S100g (Mm.PT.58.7385865), forward 5'-TCAGAGTTCCCCAGCCT-3', reverse 5'-TCCTGACTTGTTCATTGTGAGAG-3', probe 5'-CCATCGCCATTCTTATCCAGCTCCT-3'. Aliquots of cDNA (5 ul) were amplified under the following conditions: 95 ^{o}C for 3 min, followed by 40 cycles of 95 ^{o}C for 15 s, 60 ^{o}C for 45 s, 72 ^{o}C for 30 s. The fold change of gene expression for Cyp24a1, TRPV6, and S100g was determined using the $2^{-\Delta\Delta CT}$ method with normalization relative to Rplp0 level as described elsewhere (127).

3.3.6 Statistical analysis

The statistical analysis was conducted in SPSS (IBM SPSS Statistics 25, Armonk, New York). All data are presented as mean ± SEM. The normality of data was determined by the Shapiro-Wilk test and by evaluating predicted vs residual plots. When necessary, log10 or square root transformations were used to normalize the data. For experiment I, we performed independent sample t-tests to compare Ca absorption efficiency of WT to VDR KO mice at duodenum or proximal colon. For experiment II, body weight was evaluated as (a) pre-treatment body weight, (b) post-treatment body weight, and (c) the change of body weight (post – pre) among all treatment groups using one-way ANOVA. For gene expression, we first identified and excluded samples with degraded RNA quality and samples with $2^{-\Delta\Delta CT}$ values that were > 10-fold different from those within the same treatment group. We then evaluated the response of gene expression with increasing doses of Sg leaf or 1,3-diglucuronide-1,25$(OH)_2$D by fitting the gene expression data into a linear regression model (Origin 2019b, OriginLab, Northampton, MA). Furthermore, we tested which dose of either Sg leaf or 1,3-diglucuronide-calcitriol would induce an increase in mRNA expression when compared with the control group. When the F statistic in the one-way ANOVA test was significant, post hoc pairwise comparisons were made by the Tukey-Kramer test. $p<0.05$ was considered statistically significant for all analyses.

3.4 Results

We directly measured and compared Ca absorption efficiency at proximal colon and duodenum from the same mouse using an *in situ* ligated loop method (2 mM Ca, 10 min). In WT

mice, the Ca absorption efficiency at duodenum was 30.0 ± 6.7%. The proximal colon had similar absorptive capacity (26.24 ± 3.67%) (Fig. 3.1). However, age matched VDR KO mice had significantly lower Ca absorption efficiency in the duodenum (11.7 ± 4.12%, p=0.04 vs WT) and proximal colon (13.75 ± 2.83%, p=0.02 vs WT).

Prior to the dietary intervention in experiment II, body weight ranged from 19.8-22.9 g in the mice. Randomization was done to minimize the body weight difference between groups. Mice gained 0.9-2.0 g during the two weeks' dietary intervention period and neither Sg leaf nor 1,3-diglucuronide-1,25$(OH)_2$D treatment negatively affected body weight gain.

We examined whether the Sg leaf or the 1,3 diglucuronide-1,25$(OH)_2$ D could increase the expression of Cyp24a1, the most responsive vitamin D regulated gene that encodes the enzyme that initiates the degradation of 1,25$(OH)_2$D, and for genes proposed to be critical for transcellular Ca absorption: Trpv6 and S100g. Using regression analysis, we found that Sg leaf dose-dependently induced the expression of Cyp24a1 mRNA (Adj. r^2 = 0.87, p = 0.01) and Trpv6 mRNA (Adj. r^2 = 0.88, p = 0.01) in the proximal colon (Fig. 3.2A). The highest expression of Trpv6 mRNA and Cyp24a1 mRNA at proximal colon was seen with 2 ng calcitriol equivalent per day Sg leaf group (Fig. 3.2Aa, b). The synthetic compound 1,3-diglucuronide-1,25$(OH)_2$D also dose-dependently upregulated the expression of Cyp24a1 mRNA (Adj. r^2 = 0.91, p = 0.008) and Trpv6 mRNA (Adj. r^2 = 0.7, p = 0.048) (Fig. 3.2Ba, b). We also observed a dose dependent increase in S100g mRNA levels (Adj. r^2 = 0.83, p = 0.02, Fig. 2Bc) with the synthetic compound, and Trpv6 mRNA expression was significantly increased starting with 1 ng calcitriol equivalent per day (Fig. 3.2Bb).

A previous study in mice showed that 1000 µg Sg leaf per day (i.e. 5-times more than our largest dose), caused a significant increase in plasma 1,25$(OH)_2$D concentrations that induced duodenal Cyp24a1 mRNA level and raised serum Ca (147). Our doses were chosen to avoid this systemic effect so that the freed calcitriol action would be targeted to the lower bowel. As expected, even the highest dose of Sg leaf that we used had no effect on the expression of vitamin D responsive genes in the duodenum (Fig. 3.3A). In contrast, duodenal Cyp24a1, S100g and Trpv6 mRNA levels were elevated at the highest dose of the 1,3-diglucuronide-1,25$(OH)_2$D, but this difference was significant only for Trpv6 mRNA (Fig. 3.3B).

Although the glycoside forms of 1,25$(OH)_2$D from Sg leaf are not liberated in ileum, cleavage of synthetic glucuronide forms of calcitriol by bacterial glucuronidases in the ileum is

possible (147). We examined the ileum and cecum for evidence of vitamin D action to assess these off-target effects. As expected, Sg leaf had no effect on the expression of Cyp24a1, Trpv6, or S100g in either ileum or cecum (Table 3.1). In contrast, 1,3-diglucuronide-1,25$(OH)_2$D caused a dose-response effect on Cyp24a1 mRNA in the cecum ($r^2 = 0.92$, $p = 0.007$) (Table 3.1). Neither Trpv6 nor S100g mRNA levels were increased by the synthetic compound in the ileum or cecum (Table 3.1).

3.5 Discussion

Our research is important because it confirms that the proximal colon is a site for vitamin D-mediated intestinal Ca absorption and because it demonstrates that glycosylated and glucuronidated forms of 1,25$(OH)_2$D can be used to upregulate elements of the Ca absorption machinery in the proximal colon.

Most of what we know about Ca absorption is from studies on the small intestine. These studies show that 1,25$(OH)_2$D regulates a saturable, transcellular process (71) that predominates under low and adequate dietary Ca intake levels (88). Although research on intestinal Ca absorption has focused on the small intestine, the residence time for dietary Ca in duodenum is very short (2.5 min (149)), which suggests the proximal intestine may have a modest role in whole body calcium metabolism. Furthermore, other research shows that active, vitamin D-regulated Ca absorption also occurs in the lower bowel. Harrison and Harrison (150) first reported that vitamin D repletion of vitamin D deficient rats increased active Ca transport in everted sacs of rat colon. This was later confirmed by Petith et al. (151) who used an *in situ* colonic perfusion technique to study Ca absorption. Lee et al. (152) subsequently used Ussing chambers to show that 1,25$(OH)_2$D injections (3 days, 270 ng/d) could stimulate net active Ca absorption in the proximal colon of vitamin D deficient rats while Karbach and Rummel (9) showed that the proximal colon of rats exhibited saturable Ca absorption kinetics. Other research has also shown that the saturable, vitamin D-regulated Ca absorption capacity exists in the cecum (10, 13) and distal colon (11, 97, 134). However, we believe that Ca absorption in the proximal colon is likely to be more physiologically important in humans. This is because the cecum is not well developed in humans and because the solid feces in the distal colon makes it unlikely that the Ca in that segment will reach the apical membrane Ca transporters that mediate Ca uptake into the intestinal epithelial cell.

Dhawan et al. (15) recently showed that transgenic expression of VDR to the lower bowel was sufficient to prevent the abnormal calcium and bone phenotypes typically seen in VDR KO mice, thereby demonstrating that vitamin D-mediated Ca absorption in the lower bowel plays a critical *in vivo* role in calcium homeostasis. Our work extends the earlier research on Ca absorption because we are the first to directly measure and compare Ca absorption capability between proximal colon and duodenum in the same mouse *in vivo*. In doing so, we found that proximal colon (26.2 ± 3.7% of the test dose absorbed in 10 min) has a similar ability to absorb Ca as does the duodenum (30.0 ± 6.7%) (Fig. 1). In addition, while others have used the VDR KO model to establish the role of vitamin D in small intestinal Ca absorption (7, 55), we are the first to demonstrate that Ca absorption at proximal colon is also dependent on the VDR.

Given the long time dietary Ca spends in the cecum and colon (e.g. 184 min in rats (153)), the existence of vitamin D-regulated Ca absorption in the large intestine suggests this capacity could be targeted to improve Ca homeostasis. Recent research shows that glycoside forms of $1,25(OH)_2D$ in Sg leaf (147) and a synthetic 25-monoglucuronide form of $1,25(OH)_2D$ (143) can regulate gene expression in the colon without having systemic effects that influence duodenal gene expression. In the small intestine, the binding of glucuronide and glycoside moieties to $1,25(OH)_2D$ prevent it from being absorbed or activating VDR. However, in the lower bowel of monogastric animals, bacterial glycosidase and glucuronidase activity can cleave the glycoside or glucuronide from $1,25(OH)_2D$, allowing it to act locally. Unfortunately, when these modified forms of calcitriol are present at high levels, the $1,25(OH)_2D$ liberated in the lower bowel can also be absorbed and have systemic effects (154). Consistent with the liberation of calcitriol by large intestinal bacteria, the kinetics of $1,25(OH)_2D$ appearance in blood after a single, large bolus of Sg leaf is delayed and longer lasting than a comparable oral dose of $1,25(OH)_2D$ (155). The most comprehensive examination of colonic targeting with Sg leaf is the report by Zimmerman et al. (147). They fed increasing amounts of Sg leaf to mice for 10 days and found that up to 333 ug of Sg leaf per day (~3.3 ng equivalents of calcitriol) induced colonic expression of Cyp24a1 mRNA (as a marker of cellular vitamin D action) without causing systemic effects (i.e. increasing serum $1,25(OH)_2D$ levels or duodenal Cyp24a1 mRNA levels). Consistent with Zimmerman's study, we showed that a 200 ug dose of Sg leaf specifically targeted the proximal colon and induced Cyp24a1 mRNA and Trpv6 mRNA levels (Fig. 2Aa, b). Thus, while the $1,25(OH)_2D$ liberated from Sg leaf or the 1,3-diglucuronide-$1,25(OH)_2D$ stimulates the Ca absorptive machinery in the proximal

colon, it also induced the negative feedback loop for hormone degradation that is mediated by Cyp24a1. Future studies will need to determine whether the induction of Cyp24a1 limits the benefit of the colon-targeted compounds. Still, our data show that optimal levels of Sg leaf or the 1,3-diglucuronide-1,25$(OH)_2$D may have advantages over other glucuronidated forms of calcitriol. Koszewski et al. (148) previously showed that a single dose of 24 pmol of a synthetic β-25-monoglucuronide form of 1,25$(OH)_2$D could also induce both Cyp24a1 and Trpv6 mRNA in mouse colon. However, the dose they used also increased serum 1,25$(OH)_2$D levels and duodenal Trpv6 mRNA levels.

A weakness in using Sg leaf to target the colon is that it is a natural product that contains many different 1,25$(OH)_2$D glycosides (with between 1 and 10 glucose moieties attached) (155). Thus, it may be difficult to standardize the product across experiments and research labs. Although others have studied the colonic actions of a synthetic β-25-monoglucuronide form of 1,25$(OH)_2$D (143, 148), the major modified form of 1,25$(OH)_2$D in Sg leaf is a form with glycoside residues on the 1 and 3 positions on the A ring (147). Here, we tested a novel 1,3-diglucuronide of 1,25$(OH)_2$D for its effects on the proximal colon. Our study found that 1,3-diglucuronide of 1,25$(OH)_2$D not only induced a dose-dependent increase of Trpv6 mRNA expression, but also S100g mRNA expression, in the proximal colon (Fig. 2B). However, the highest dose of 1,3-diglucuronide-1,25$(OH)_2$D also increased duodenal expression of Trpv6 mRNA (Fig. 3Bb), indicating it had systemic effects. Previous research on β-25-monoglucuronide-1,25$(OH)_2$D shows that calcitriol can be liberated *in vitro* by bacterial glucuronidases within the luminal contents of the ileum (143). Our observation that 1,3-diglucuronide-1,25$(OH)_2$D induces cecal Cyp24a1 mRNA suggests it may also be liberated earlier than the glycosylated forms of calcitriol in Sg leaf (Table 1). In addition, the induction of cecal Cyp24a1 mRNA by 1,3-diglucuronide 1,25$(OH)_2$D may degrade the freed 1,25$(OH)_2$D and limit its beneficial effects in the proximal colon.

In summary, our studies reveal important facets of colon Ca metabolism. First, we clearly show that the proximal colon has a physiologically relevant and vitamin D sensitive capacity to absorb Ca that is similar to the well-studied capacity of the duodenum. In addition, we have demonstrated that glycosylated and glucuronidated forms of calcitriol can target the vitamin D-sensitive Ca absorption machinery in the proximal colon. Although the glycosylated forms of calcitriol and 1,3-diglucuronide-1,25$(OH)_2$D can both target the proximal colon, the 1,3-diglucuronide-1,25$(OH)_2$D may cause systemic effects that will require careful dose optimization.

Our work is significant because the ability to activate Ca absorption in the proximal colon could be beneficial for people with poor bone health due to compromised intestinal Ca absorption capacity. For example, targeting Ca absorption in the lower bowel could help people who have undergone bariatric surgery procedures like Roux-en-Y gastric bypass (RYGB) that reduce Ca absorption in human (136, 156, 157) and animals (137, 138) and contributes to reduced BMD in these patients (139). However, additional studies are needed to directly test whether vitamin D-mediated gene expression induced by Sg leaf and/or 1,3-diglucuronide-1,25$(OH)_2$D can promote Ca absorption in the proximal colon.

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3.6 Figures and Table

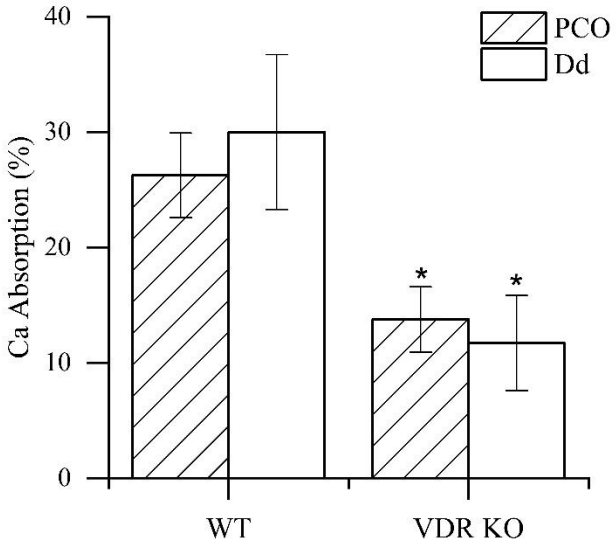

Figure 3.1. Ca Absorption Efficiency (%) is Significantly Reduced in Proximal Colon and Duodenum of VDR KO Mice

Weanling VDR knockout (KO) mice were fed rescue diet and age-matched wild type (WT) mice were fed AIN93G diet. At 7 to 10-week-old, Ca absorption efficiency was measured simultaneously in the proximal colon (PCo) and duodenum (Dd) of the mice using *in situ* ligated loops (2 mM Ca, 10 min). Data are expressed as mean ± SEM (n = 5-8 per group for duodenum; 8-10 per group for proximal colon). *Significantly different from WT ($p < 0.05$, t-test).

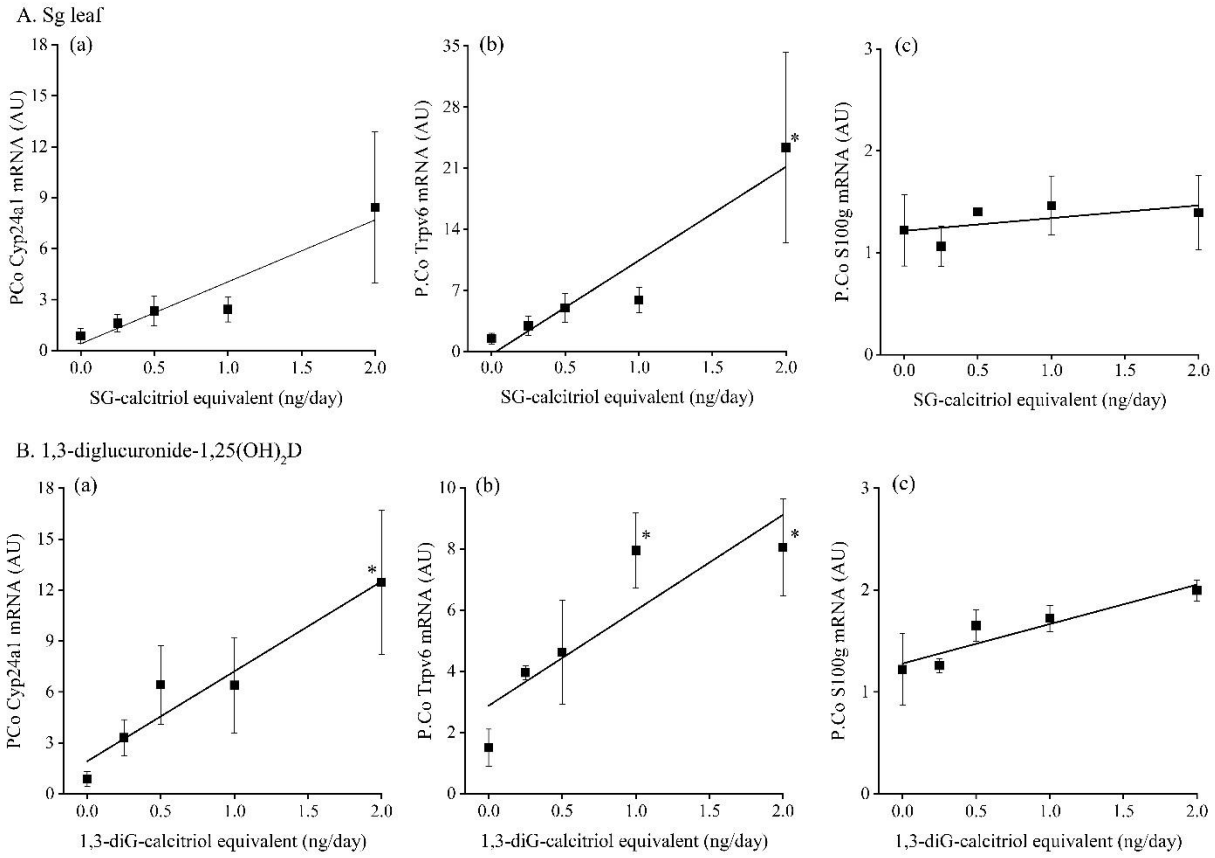

Figure 3.2. Both Sg leaf and 1,3-diglucuronide-1,25(OH)$_2$D Dose-Dependently Induced Expression of Vitamin D Target Genes in the Proximal Colon.

Three-month-old female mice were fed AIN93G-based diets containing increasing amounts of (A) Sg leaf or (B) 1,3-diglucuronide-1,25(OH)$_2$D for two weeks. Doses were calculated to provide a specific dose of calcitriol. RNA was isolated from the proximal colon (PCo) and mRNA was examined for (a) Cyp24a1, (b) Trpv6, and (c) S100g by real-time PCR. AU: arbitrary unit. Data are expressed as mean ± SEM (n = 3-5 per group). Dose response relationships were determined using Pearson's correlation. * Significantly different from the control group ($p < 0.05$, Tukey-Kramer test).

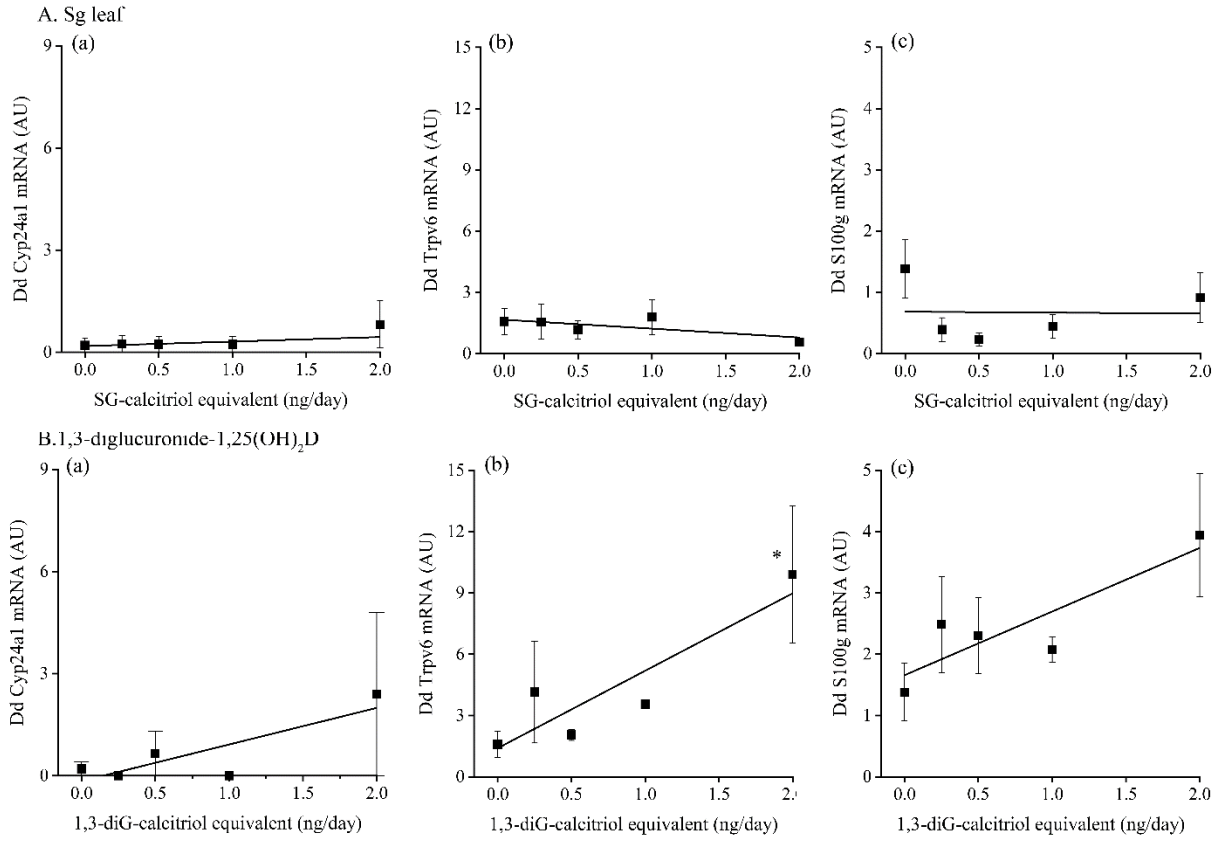

Figure 3.3. Sg leaf and 1,3-diglucuronide-1,25(OH)$_2$D Have Limited Effects on Expression of Vitamin D Target Genes in the Duodenum

Three-month-old female mice were fed AIN93G-based diets containing increasing amounts of (A) Sg leaf or (B) 1,3-diglucuronide-1,25(OH)$_2$D for two weeks. Doses were calculated to provide a specific dose of calcitriol. RNA was isolated from the duodenum (Dd) and mRNA was examined for (a) Cyp24a1, (b) Trpv6, and (c) S100g by real-time PCR. AU: arbitrary unit. Data are expressed as mean ± SEM (n = 3-5 per group). Dose response relationships were determined using Pearson's correlation. * Significantly different from the control group ($p < 0.05$, Tukey-Kramer test).

Table 3.1. The Effect of Sg Leaf or 1,3-diglucuronide-1,25$(OH)_2$D on Expression of Vitamin D Target Genes in the Ileum and Cecum

Three-month-old female mice were fed AIN93G-based diets containing increasing amounts of Sg leaf or 1,3-diglucuronide-1,25$(OH)_2$D for two weeks. Doses were calculated to provide a specific dose of calcitriol (ng/day). RNA was isolated from the ileum and cecum after which mRNA was examined for Cyp24a1, Trpv6, and S100g by real-time PCR. Data are expressed as mean ± SEM (n = 2-5 per group). * Significantly different from the control group ($p < 0.05$, Tukey-Kramer test).

		Ileum		Cecum	
mRNA	Dose (ng/day)	Sg Leaf	1,3 diG	Sg Leaf	1,3 diG
Cyp24a1	0	1.65 ± 0.84 (5)	1.65 ± 0.84 (5)	1.16 ± 0.31 (5)	1.16 ± 0.31 (5)
	0.25	0.06 ± 0.03 (5)	1.66 ± 1.36 (2)	2.94 ± 1.24 (5)	2.24 ± 0.61 (5)
	0.5	2.80 ± 1.47 (5)	1.64 ± 0.95 (4)	1.81 ± 0.55 (5)	3.56 ± 0.94 (5)*
	1	3.28 ± 1.89 (4)	2.43 ± 0.95 (5)	3.35 ± 1.23 (5)	8.17 ± 2.04 (5)*
	2	0.73 ± 0.29 (5)	4.57 ± 1.37 (3)	2.75 ± 0.98 (3)	10.63 ± 3.13 (4)*
Trpv6	0	0.74 ± 0.45 (5)	0.74 ± 0.45 (5)	2.05 ± 1.44 (5)	2.05 ± 1.44 (5)
	0.25	1.77 ± 0.8 (5)	0.49 ± 0.32 (5)	2.09 ± 0.62 (5)	0.54 ± 0.32 (5)
	0.5	0.15 ± 0.15 (5)	1.08 ± 0.47 (5)	1.70 ± 0.51 (5)	0.05 ± 0.01 (5)
	1	2.04 ± 1.41 (5)	0.58 ± 0.29 (5)	1.96 ± 0.29 (5)	0.15 ± 0.06 (5)
	2	0.59 ± 0.3 (3)	0.20 ± 0.2 (5)	2.71 ± 0.72 (5)	0.06 ± 0.02 (5)
S100g	0	1.19 ± 0.39 (5)	1.19 ± 0.39 (5)	1.32 ± 0.56 (5)	1.32 ± 0.56 (5)
	0.25	0.41 ± 0.22 (5)	3.51 ± 1.02 (5)	1.32 ± 0.29 (5)	1.22 ± 0.36 (5)
	0.5	0.76 ± 0.28 (5)	1.54 ± 0.58 (5)	1.40 ± 0.41 (5)	1.55 ± 0.28 (5)
	1	2.06 ± 0.96 (5)	1.71 ± 0.35 (5)	1.31 ± 0.14 (5)	1.80 ± 0.45 (5)
	2	0.46 ± 0.1 (5)	4.27 ± 1.65 (5)	2.01 ± 0.45 (5)	1.66 ± 0.71 (5)

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CHAPTER 4. TRANSCRIPTOMICS ANALYSIS OF AGE-ASSOCIATED INTESTINAL RESISTANCE TO 1,25(OH)$_2$D ACTION

4.1 Abstract

During aging, intestinal calcium (Ca) absorption declines even while serum 1,25(OH)$_2$D level increases, suggesting the existence of age-related resistance to 1,25(OH)$_2$D action. Some studies suggest that age-related reductions in intestinal VDR level is the cause of the age-related resistance to 1,25(OH)$_2$D action, suggesting the resistance is due to disruption in VDR-mediated gene expression. However, the genome wide impact of age on the 1,25(OH)$_2$D action in the intestine has not been studied. We tested the hypothesis that age is associated with reduction in 1,25(OH)$_2$D-regulated transcripts across the genome. 4-month old (mature), 11.5-month old (middle-aged), and 20.5-month old (old) female C57BL/6J mice (n=20/age, balanced by gender) were fed a modified AIN93G diet (0.8% strontium, 0.02% Ca, 0.36% P) for 2 weeks to inhibit renal 1,25(OH)$_2$D synthesis.. Afterwards, mice were treated with vehicle or 1,25(OH)$_2$D (i.p. 0.5 ng/g BW, 4h). Duodenal scraping was harvested for RNA extraction and RNA-sequence (RNA-seq) analysis.

The total number of 1,25(OH)$_2$D-regulated differentially expressed genes (DEG) is the highest in the mature female mice. Although some DEG are in all age groups, we found 33, 2, and 14 1,25(OH)$_2$D-responsive genes that are unique to the mature, middle-age, and old group, respectively. To make sure the age-associated difference in DEG list was not affected by a conservative false discovery rate (FDR), we raised the FDR to 20% and found it has minimal effect. Additionally, we found significant overlapping between our DEG list and a published RNA-seq report that studied 1,25(OH)$_2$D-regulated transcripts in the proximal intestine of Cyp27b1 knockout mice. Next, we assessed the effect of age on the fold induction of expression for 1,25(OH)$_2$D-regulated genes. While the fold induction for some genes (Cyp24a1, Trpv6) declined in the old versus the mature or middle-aged mice, some genes were unaffected by age. Furthermore, we did not observe an effect of age on Vdr mRNA level. Since we cannot evaluate the effect of age in male mice, we did a preliminary examination to assess whether sex might be a significant modifier for the impact of age on 1,25(OH)$_2$D action using middle-aged mice. We found there are more 1,25(OH)$_2$D-regulated DEG in male than female. However, while the fold induction of expression for some genes (e.g., S100g) is higher in male, others (e.g., Cyp24a1, Trpv6) is higher

in female. Our findings falsified the hypothesis of a universally present resistance to $1,25(OH)_2D$ action due to reduced VDR level. Additionally, sex-related differences in intestinal responses to $1,25(OH)_2D$ emphasized the necessity for future research to be done on male mice.

4.2 Introduction

Intestine is part of the three-tissue axis that maintains calcium (Ca) homeostasis, the disruption of which leads to hypocalcemia and causes severe health outcomes. When dietary Ca intake is low, Ca homeostasis is maintained through enhanced intestinal Ca absorption, renal Ca reabsorption, and bone Ca resorption. Higher intestinal Ca absorption efficiency can reduce Ca loss from bone under dietary Ca restriction (158, 159). Intestinal Ca absorption is not only positively associated with higher peak bone mass and better bone microstructure during growth (1, 2, 119), but also contributes to reduced adult bone loss (44, 140). An average of 4.8-year follow up of 5452 women (>= 69 years old) found that when dietary intake is less than 400 mg/d, women with lower Ca absorption efficiency had a 2.5-fold increase in hip fracture risk (120). Given the key role of intestinal Ca absorption in bone health during growth and in adulthood, it is of vital importance to understand how it is regulated.

Over 70 years ago, people first observed the dependence of intestinal Ca absorption on $1,25(OH)_2D$, the active form of vitamin D (46). Since then, mechanistic studies found that $1,25(OH)_2D$ and vitamin D receptor (VDR) mediate the active transcellular Ca absorption by transcriptionally regulating the expression of several key genes (e.g., Trpv6, S100g) (7, 55). When $1,25(OH)_2D$/VDR signaling was disrupted during growth in VDR KO mice, intestinal Ca absorption, serum Ca, and bone phenotypes (e.g., femur length and density) were abnormal (8). However, villin promoter-mediated intestine-specific VDR transgene expression restored VDR KO-induced abnormalities. These observations suggest that the regulation of intestinal Ca absorption is the single most important role for $1,25(OH)_2D$/VDR signaling during growth.

Different from the significant role $1,25(OH)_2D$ signaling plays in Ca metabolism for the growing intestine, intestine develops resistance to $1,25(OH)_2D$ action with age. In 1991, using data collected from 51 healthy women 26-88 years old, Eastell et al. (115) observed an increase of $1,25(OH)_2D$ level with age (+38%, $r=0.40$, $p<0.01$) while fractional Ca absorption remain unaltered, suggesting resistance to $1,25(OH)_2D$ actions with age in the intestine. Similar observations were also made by others (30). Later, Wood et al. (26) formally tested the effect of

age on intestinal responsiveness to 1,25$(OH)_2$D actions across a wide range of serum 1,25$(OH)_2$D levels. Young (3-month old) and old (23-month old) male rats were treated with vehicle or various doses of 1,25$(OH)_2$D infusion (4 ng, 14 ng/100g BW/day) for 9 days. When the association between duodenal Ca absorption efficiency and plasma 1,25$(OH)_2$D level (<400 pg/ml) was fitted to a linear regression, the response of old rat intestine to increased 1,25$(OH)_2$D was about half of that seen in the young rat intestine (ratio of linear regression slope = 0.46). A similar extent of impaired intestinal responsiveness of Ca absorption to a wide range of 1,25$(OH)_2$D levels was also observed in a study comparing elderly (72.5 ± 3.0 year) and young women (28.7 ± 5.3 year) (ratio of linear regression slope = 0.5) (27). These data indicates that advanced age causes intestinal resistance to 1,25$(OH)_2$D. Although this phenomenon was discovered a few decades ago, the exact molecular mechanism underlying this phenomena is still uncertain and it is unclear whether age-associated intestinal vitamin D resistance affects all vitamin D target genes equally. Some evidence suggests that reduced VDR level is a contributing factor as intestinal VDR level has been observed to decrease with advancing age in human and animal model (28, 30, 160). However, others did not find an aging-related reduction in intestinal VDR level (26). Therefore, the effect of age on intestinal resistance to 1,25$(OH)_2$D on a bigger genome scale and its association with intestinal VDR level should be investigated.

The goal of our study is to systematically examine three age groups of mice (i.e., mature, middle-aged, old) and investigate the full scope of age-associated intestinal resistance to 1,25$(OH)_2$D action. To achieve our goal, we will conduct an RNA-sequence (RNA-seq) analysis of 1,25$(OH)_2$D regulated gene expression in the small intestine of mice. Our primary hypothesis is that advanced age is associated with intestinal resistance to the molecular action of 1,25$(OH)_2$D and that this will be reflected in a reduced induction of 1,25$(OH)_2$D-responsive transcripts in old mice vs mature or middle-aged mice. The primary hypothesis will be examined by comparing the number of 1,25$(OH)_2$D-responsive differentially expressed gene(s) (DEG) and the fold induction of DEG across age groups. Our research will potentially help to develop therapeutic strategies to improve the intestinal responsiveness to 1,25$(OH)_2$D and reduce bone loss and osteoporosis risk in the elderly.

4.3 Methods

4.3.1 Study design

C57BL/6J mice (The Jackson Laboratory) of three age groups: 4-mo-old (mature), 11.5-mo-old (middle-aged), and 20.5-mo-old (old) (n=20/age, balanced by gender) were fed a modified AIN93M diet (0.5% Ca, 0.4% P, 200 IU VD_3, Research Diet) upon arrival during a 10-day acclimation period. Mice were then fed a modified AIN93M diet with high strontium and low calcium (0.8% strontium, 0.02% Ca, 0.36% P, Research Diet) for 2 weeks to inhibit the endogenous renal synthesis of $1,25(OH)_2D$. Afterwards, mice were randomized to either receive a single dose of vehicle (a 1:9 mixture of ethanol and propylene glycol, 2 µl/g BW) or $1,25(OH)_2D$ (i.p. 0.5 ng/g BW in vehicle). Injection solutions also contained coomasie blue as a means to evaluate the quality of the injection (i.e. a proper injection will result is a diffuse blue peritoneal fluid). 4 hours after the injection, mice were euthanized. Mucosal scrapings from the duodenum were harvested and homogenized in ice-cold TRI Reagent (Zymo Research, Irvine, CA) then immediately frozen in liquid nitrogen until analysis.

4.3.2 Tissue and RNA processing

RNA from intestine was isolated using Direct-zol RNA Miniprep Plus Kit (Zymo Research, Irvine, CA) according to the manufacturer's directions with two modifications. First, we used the aqueous layer of the TriReagent homogenate that results from addition of 1-Bromo-3-chloropropane to the sample. Second, we washed the column a second time with 0.7 ml of 80% ethanol to remove residual salts that lower the A260/230 ratio. The isolated RNA was eluted in 50 µL DNase/RNase-Free water and quantified using a NanoDrop Spectrophotometer 2000 (ThermoFisher Scientific). RNA degradation and contamination were checked by agarose gel electrophoresis (1.5 %, 100 V, 30 min). Isolated RNA samples were stored in -80 ^{0}C until the analysis of differential gene expression by RNA-seq.

4.3.3 RNA-seq analysis

Duodenal RNA samples from each age group (n=20/age, balanced by gender) were subjected to RNA-seq analysis by Novogene (CA, USA). RNA integrity was assessed using an

Agilent Bioanalyzer 2100 System (Agilent Techniologies, CA). A RNA integrity number (RIN) (range 1-10) was generated for each sample with higher number indicates better quality of RNA. Of our 60 samples, 45 had a RIN score > 6.5, 12 more had a score < 6, and three had a score between 5.5 and 6.0. All 60 RNA samples were used for downstream sequencing analysis.

A total of 1 ug RNA per sample was used for preparing RNA-seq library with NEBNext® UltraTM RNA Library Prep Kit for Illumina® (New England Biolabs, MA). Library quality was assessed on the Agilent Bioanalyzer 2100 system. The library was subjected to Illumina next generation sequencing (Illumina NovaSeq 6000) and at least 20 million paired-end reads were generated (150-bp read length). Reads were mapped to reference genome (Ensembl mm10) using the Spliced Transcripts Alignment to a Reference (STAR) software (v2.5). HTSeq v0.6.1 was used to count the reads mapped of each gene. Read counts were normalized and differential expression analysis between two groups was performed using the DESeq2 R package (v2_1.6.3). The resulting P values were adjusted using the Benjamin and Hochberg's approach for controlling the False Discovery Rate (FDR). Genes with an adjusted P value ≤ 0.1 found by DESeq2 were considered as differentially expressed. Genes are defined as non-present if the raw counts between two groups for comparison are both lower than 10. Otherwise, genes are considered present. For visualization, data was expressed as fragments/kb of exon per million mapped (FKPM).

4.3.4 Generation of heat map

To assess the consistency of responses across the replicates within each group we conducted hierarchical clustering of the top 100 1,25$(OH)_2$D regulated genes using GenePattern (161). Data was normalized and mean centered for each gene (i.e. by row) through hierarchical clustering to reveal patterns in the 1,25$(OH)_2$D regulated genes. A heat map was generated for visualization.

4.3.5 Compare our DEG List to published data

To gain confidence that our DEG list is accurate, we compared our results to data from a published report that examined the impact of 1,25$(OH)_2$D treatment on intestinal gene expression in Cyp27b1 knockout mice (79). The full report and data can be accessed from here (79). In Excel, we identified the common genes between Lee's DEG list and our DEG list by highlighting the

duplicates of gene names between two lists. The number of common genes between Lee's DEG list and each of our age specific DEG list was summed up and presented in Venn diagram.

4.4 Results

4.4.1 Sequencing and mapping

The quality of the sequencing were assessed by three parameters: error rate, GC content distribution, and composition of raw reads. Our sequencing error rate is below 0.03%, which is much lower than the generally acceptable rate of 1%. GC content distribution is an indicator of potential AT/GC separation, which affects gene expression quantification. Given random fragmentation and biological law of G/C-A/T content, the content of G/C, A/T should be respectively equal. Our 60 samples have relatively equal content of A/T and G/C (G/C content ranges from 48-50%). Lastly, the composition of raw reads was assessed and our samples have high percentage of clean reads (96-98%). Furthermore, to ensure only clean reads were used for gene expression quantification, reads with low quality were removed from the raw reads based on these criteria: a) reads with adaptor contamination, b) reads with uncertain nucleotides constitute more than 10% of either read (N > 10%), c) reads with low quality nucleotides (Base Quality less than 5) constitute more than 50% of the read. As a result, we have at least 20 million total clean reads per sample used for downstream analysis.

When clean reads were mapped to the reference genome, the total mapped rate is $\geq$ 94.91% per sample. Over 88% of mapped reads per sample were mapped to the exon region of the reference genome.

4.4.2 Evaluation of the quality of 1,25(OH)$_2$D treatment

A total of 15,823 genes were present in 1,25(OH)$_2$D and control treated male and female mice. Among female mice, the total number of present genes is: 15,962 for the mature, 16,292 for the middle-aged, and 16,285 for the old group.

Across all three age groups and all sex groups we identified 100 potential vitamin D-regulated differentially expressed genes. We conducted hierarchical clustering on this group of 100 genes using all 60 samples and visualized these findings using a heat map (supplemental Fig. 4.1). In the map, vehicle (on the left half) and 1,25(OH)$_2$D treated mice (on the right half) were

separated by their level of gene expression in response to the corresponding treatment. From the top of the heat map, 122 genes were upregulated in 1,25$(OH)_2$D treated group and their high level of expression is indicated by the red color in the map. In comparison, the vehicle treated control mice has low level of expression for these genes as indicated by the blue color. Towards the bottom of the heat map, the expression of 83 genes were downregulated in the 1,25$(OH)_2$D treated mice relative to the control mice.

Although the overall gene expression pattern reflects the expected response to 1,25$(OH)_2$D or vehicle for each group. The heat map revealed several problems that affected the data for male mice in our dataset. First, 4 out of total 5 samples in the 1,25$(OH)_2$D treated, old male group (O6, O7, O8, O9) had gene expression levels that were more consistent with the expression seen in the control mice. This is consistent with our observation during harvest that the injection was localized into the seminal vesicle of these old male mice. This was unanticipated as the old mice all had enlarged seminal vesicles. Additionally, one sample from the vehicle treated old male sample (O3) had gene expression levels that were higher than the other mice in that group. Lastly, three of the vehicle treated young male samples (Y1, Y4, Y5) displayed a pattern of higher gene expression than what we observed for control mice in other age and sex groups. As a result, only 2 genes that were differentially expressed by 1,25$(OH)_2$D in the young male group, suggesting compromised sample quality. Because of our concerns regarding the quality of samples from male mice in the young and old groups, we decided to exclude these groups from our analysis.

4.4.3 The effect of advanced age on 1,25$(OH)_2$D actions in the duodenum of female mice

4.4.3.1 The effect of advanced age on the number of 1,25$(OH)_2$D regulated genes

We evaluated the effect of advanced age on duodenal 1,25$(OH)_2$D actions in female mice. First we asked if advanced age reduces the total number of 1,25$(OH)_2$D-regulated genes in the duodenum. Our DeSeq2 analysis showed that 1,25$(OH)_2$D treatment regulated a total of 200 transcripts (129 upregulated, 71 downregulated) in an analysis that combined samples across the age groups (n=15/treatment) (Table 4.1). We also found age-specific differences in the vitamin D-regulated DEG list i.e. mature = 80, middle-age = 30, and old = 35 (n=5/treatment). Some genes are differentially expressed in more than one age groups. Additionally, a large proportion of the 1,25$(OH)_2$D-regulated genes from the individual age DEG list overlaps with the combined DEG

list. For example, 47 out of 80 DEG in the mature group were also regulated by 1,25$(OH)_2$D in the combined analysis and 87.2% of these genes were upregulated in both groups. Although our comparison of age groups showed that several of these were robust enough to be detected as DEG in the combined analysis (Fig. 4.1a), there are genes that appear to be unique to each age group (Fig. 4.1b). 33 gene in mature, 2 genes in middle-aged, and 14 genes in old mice were not in either the combined analysis or in another age group list (Fig. 4.1b). This suggests that these genes are the uniquely age-specific 1,25$(OH)_2$D DEG. Collectively, these results suggest that advanced age has a negative effect on the total number of 1,25$(OH)_2$D-regulated genes in female mice.

A possible reason for poor overlap in the DEG lists across age groups is that the adjusted p value for a gene in one age group may be higher than our selected FDR cut off. For example, at 10% FDR threshold, the existence of samples with weak adjusted p value (e.g., 0.11) will lead to the conclusion that there is difference in the number of DEG across age. We tested whether we could capture more overlap in the DEG lists across age by raising the significance threshold to 20% FDR. As shown in Table 4.2, raising the FDR to 15% or 20% has a negligible effect on our DEG list; the number of 1,25$(OH)_2$D regulated DEG in the mature mice is still much higher compared to the middle-aged and old mice.

1,25$(OH)_2$D treatment in the intestine of 8-week old Cyp27b1 knockout mice regulated 719 unique transcripts as DEG (79). A total 40 genes that were differentially expressed in any of our age groups were also in Lee's DEG list (Table 4.3): 28 from mature, 20 from middle-aged, and 13 from old. The majority of these overlapping genes were differentially expressed in at least 2 age groups (Fig. 4.2). Among the 8 genes that were differentially expressed in all 3 age groups, 5 of them were also in Lee's DEG list and they were all upregulated by 1,25$(OH)_2$D.

4.4.3.2 The effect of advanced age on the fold change of 1,25$(OH)_2$D regulated genes

Another way that advanced age could influence duodenal 1,25$(OH)_2$D actions is to reduce the magnitude of 1,25$(OH)_2$D-action. We tested this in several ways. First, we examined the impact of age on four known 1,25$(OH)_2$D target genes: Cyp24a1, Trpv6, S100g, and Slc30a10 (Fig 4.3, Table 4.4). Although the maximum 1,25$(OH)_2$D-induced level of Cyp24a1 mRNA was highest in the middle-aged group (Fig 4.3A), the fold change declines by 32.2% from maturity to the old and by 24.25% from middle-age to the old. Trpv6 mRNA induction by 1,25$(OH)_2$D was highest in the mature mice while it was lower in the middle-aged and the old mice (Fig. 4.3C) and

the fold change of Trpv6 mRNA was reduced from maturity to the old by 65.24%. In contrast, the basal and 1,25$(OH)_2$D-induced level for Slc30a10 was similar across all age groups (Fig. 4.3B) while the basal level for S100g was higher in the mature group versus the other two groups (Fig. 4.3D). Thus, neither Slc30a10 nor S100g was negatively affected by age. We also assessed the effect of age on the fold change of 6 genes that were newly identified as 1,25$(OH)_2$D-regulated across all age groups in our analysis (Table 4.4). Similar to what we observed in Cyp24a1, the fold change of Extl1 in response to 1,25$(OH)_2$D in the old group decreased compared to mature and middle-aged mice (-20%, -13.85%, respectively). Aging from maturity to old also reduced the fold change of Vstm5 (-22.76%). However, the impact of advanced age on the responsiveness to 1,25$(OH)_2$D was minimal for Pgap1, Pdp1, 5031439G07Rik, and Gm29374. Collectively, these results suggest that although the response of the duodenum to 1,25$(OH)_2$D action might vary with age, this effect not universally present across all vitamin D target genes.

4.4.3.3 The effect of age on intestinal Vdr gene expression

We assessed whether aging had an impact on intestinal Vdr mRNA level. We found that Vdr mRNA level was not regulated by 1,25$(OH)_2$D in any age group In addition, age had no effect on the basal Vdr mRNA level (Fig. 4.4).

4.4.4 The effect of advanced age on the basal level of gene expression in the duodenum of female mice

Although we have evaluated the effect of age on 1,25$(OH)_2$D-regulated gene expression, the interpretation of our data might be confounded by pre-existing age-associated differences in basal gene expression level across genome (e.g. similar to what we observed for S100G, Fig. 4.3D). Therefore, we asked whether there is any fundamental difference in basal gene expression by age. To address this question, we compared the 3 age groups of female mice for differential gene expression. As shown in Table 4.5 and Fig. 4.5, basal genes were not strongly affected in the duodenum by age. Among the 56 genes that were differentially expressed in any age groups, four genes were regulated by 1,25$(OH)_2$D at 10% FDR, five at 15% FDR and six at 20% FDR. We then individually examined the impact of 1,25$(OH)_2$D on the expression of these genes across age and found age only affected 1,25$(OH)_2$D-regulated expression for Trpv6 mRNA. Therefore, we

conclude that age-associated differences in basal gene expression isn't a major confounding factor to the effect of age on 1,25$(OH)_2$D-regulated gene expression.

4.4.5 Sex differences in basal or 1,25$(OH)_2$D-induced intestinal gene expression

Previously we reported that there are sex-specific differences in the response of young mice to 1,25 D treatment (162). However, because of the poor treatment effect in the old male group, we were not able to evaluate our primary hypothesis in males. Using the data from middle-aged male mice, we made two preliminary examinations to evaluate whether sex might be a significant modifier of intestinal vitamin D action or the impact of age on vitamin D action. First, we examined whether there were sex-specific differences in the expression of intestinal transcripts, especially those that we identified as 1,25$(OH)_2$D-regulated in female mice. In middle-aged mice, 72 genes were differentially expressed in male versus female (55 genes upregulated and 17 downregulated) (Table 4.6). However, none of these genes were identified as 1,25$(OH)_2$D-regulated in female mice.

We next examined whether sex affects the induction of genes by vitamin D. As shown in Table 4.7, 1,25$(OH)_2$D treatment resulted in a higher number of DEG in middle-aged male than female mice. Over 75% of the genes on both DEG lists were upregulated by 1,25$(OH)_2$D (Table 4.7). 35 differentially expressed genes were unique to male and 12 genes were unique to female mice (Fig. 4.6). 18 genes upregulated by 1,25$(OH)_2$D in both sexes include Cyp24a1, Trpv6, S100g, and Slc30a10 (Table 4.8). The fold induction of Cyp24a1 and Trpv6 mRNA by 1,25$(OH)_2$D was higher in female than in male mice. In contrast, the fold induction of S100g was higher in male than in female mice while sex had no effect on the induction of Slc30a10. For the remaining 10 genes listed in Table 4.9, the 1,25$(OH)_2$D-induced level and/or fold change for each gene was not different in male versus female mice except Gm29374, however, the biological function of this gene is unknown.

Collectively, these findings suggest there are gender-associated differences in 1,25$(OH)_2$D action in the duodenum of middle-aged mice that may impact the generalizability of our findings on age-associated differences in 1,25$(OH)_2$D action that we obtained using female mice.

4.5 Discussion

More than 30 years ago, people observed the phenomena in the elderly that Ca absorption efficiency reduces even though serum $1,25(OH)_2D$ level increases (30, 115). Because of these observations, people then proposed that aging causes intestinal resistance to $1,25(OH)_2D$ action which was later formally proven by two separate experiments on elderly rats and human subjects (26, 27). Some evidence suggests that intestinal resistance to $1,25(OH)_2D$ action is related to reduced intestinal VDR level, suggesting the resistance is due to disruptions of VDR-mediated intestinal gene expression in a uniform manner. However, this has never been tested. Our study filled this gap in the literature by conducting a transcriptomic analysis on $1,25(OH)_2D$-regulated genes for mature, middle-aged, and old female mice. We hypothesized that age has a universal negative effect on $1,25(OH)_2D$-regulated genes in the duodenum of female mice. Consistent with our hypothesis, we observed age-specific reduction in the vitamin D-regulated DEG list, i.e. mature = 80, middle-age = 30, and old = 35. However, the fold induction of gene expression falsified our hypothesis of a universal reduction across all genes. Among the genes that are known to be involved in intestinal Ca absorption and vitamin D metabolism, the response of Cyp24a1 (-32.2%) and Trpv6 (-65.2%) to $1,25(OH)_2D$ both decreased in the old mice compared to the mature and/or middle-aged mice. In contrast, a highly $1,25(OH)_2D$-responsive gene, Slc30a10 (163), did not show any sign of reduced responsiveness with aging and neither did S100g. 6 other newly identified $1,25(OH)_2D$-responsive genes also suggest no effect of age on their responses to $1,25(OH)_2D$. Therefore, we conclude that aging does not cause a universal resistance to $1,25(OH)_2D$.

Furthermore, we found no change in Vdr mRNA level with age, suggesting the current belief that reduced VDR level in the intestine being the cause of age-associated resistance to $1,25(OH)_2D$ is incorrect. Our finding is consistent with a previous study by Song et al. (164). They found that while a 50% reduction in intestinal VDR among 90-day old VDR KO heterozygous mice led to reduced Ca absorption in the intestine, it had no impact on the response of Trpv6 or S100g to $1,25(OH)_2D$. Collectively, these evidence indicates there might be alternative mechanisms other than intestinal Vdr expression that lead to the age-associated, non-universal resistance to $1,25(OH)_2D$ action. Others have demonstrated that VDR binding sites are distributed across large distances and these distances can vary from gene to gene. For instance, the VDR binding sites for Cyp24a1 gene is located at both near the proximal promoter region and in

intergenic regions +35, +37, +39, and +41 kb downstream of the mouse Cyp24a1 gene transcription start site (TSS) (165). In comparison, the VDR binding sites for Trpv6 was found to be -2 and -4 kb upstream of the TSS (166). Therefore, it is possible that aging might change the binding of VDR to the various binding sites for different genes. Additionally, Dwivedi et al. (167) found that the induction of Cyp24a1 gene by $1,25(OH)_2D$ (especially at low level, e.g., 10^{-10} M) depends not only on the binding of VDR to its binding site VDRE in the promoter region of Cyp24a1, but also on the binding of another transcription factor, Ets-1 to its binding site in the adjacent promoter region. Hence, it's possible that genes with transcriptional synergy as seen for Cyp24a1 are more likely to be affected by age than others without this regulation mechanism. However, future research needs to be conducted to determine the causal relationship between these mechanisms and the aging-associated various responses to $1,25(OH)_2D$.

Our study is unique because this is the first study that evaluated the effect of aging on intestinal $1,25(OH)_2D$ action on a genome scale. Some studies evaluated the effect of aging on $1,25(OH)_2D$-induced gene expression when in fact they used rapid growing animals and without including an old age group (168-170). For example, Brown et al. (169) only focused on 2-month and 12-month old rats. Although they found a reduced induction of duodenal Trpv6 in the 12-month old rats compared to the 2-month old rats, their interpretation on the effect of aging could be confounded by comparing with the rapid growing rats who are highly responsive to $1,25(OH)_2D$ signaling. Second, they left out the old age group, therefore, the evaluation of the effect of "aging" is incomplete. Additionally, these studies did not fully capture the scope of the effect of age on intestinal resistance to $1,25(OH)_2D$ on a genome scale. In our study, we evaluated the effect of aging by using three post-growth age groups: 4.5-month old mature adult mice, 11.5-month old middle-aged mice, and 20.5-month old mice. Not only our findings reflect the effect of aging without being confounded by the rapid growth group, our age groups are directly comparable to the age categorization in human. The corresponding age in human for our mice age groups are: 4.5-month old mice is equivalent for 20-30 years old in human; 11.5-month old mice is equivalent for 38-47 years in humans; and 20.5-month old mice is equivalent for humans of 56-69 years (171). Furthermore, although some people have conducted the transcriptomic analysis in the duodenum of mice, their study did not evaluate the effect of aging on intestinal response to $1,25(OH)_2D$. For example, Kazakevych et al. (172) isolated the enterocytes from the small intestine of 33-week middle-aged male mice and 118-week old male mice and compared the basal DEG. They found 7

age-upregulated and 5 age-downregulated genes, suggesting minimal effect of age on the basal transcripts. This is consistent with our conclusion about the effect of age on the basal transcripts in the duodenum of female mice: 1 gene was upregulated and 1 was downregulated for our old vs middle-aged comparison.

There are several strengths in our study. First, the effect of age on $1,25(OH)_2D$-action in the intestine was tested by evaluating not only the total number of $1,25(OH)_2D$-regulated DEG but also the fold induction of expression of these genes. Second, we enhanced our confidence in our findings by taking several steps to make sure our DEG list is accurate. For example, we raised the FDR to make sure we didn't exclude genes as DEG in the older groups because they had more variable expression and were close to being significant. We also compared our DEG list to a published we a published RNA-seq report that studied $1,25(OH)_2D$-regulated transcripts in the proximal intestine of Cyp27b1 knockout mice (79). They discovered a total 719 $1,25(OH)_2D$-regulated DEG while we found 114. However, 40 genes that were differentially expressed in both lists. The fact that our DEG list has fewer genes than Lee's and two lists did not overlap completely are probably due to a few key differences in two study design. First, they used rapid growing young (8-week old) mice that are much more responsive to $1,25(OH)_2D$ signaling than any group of our post-growth mice. Second, they used Cyp27b1 KO mice and fed the mice to a vitamin D deficient diet, both resulted in an extreme vitamin D deficient condition for the mice. Therefore, when these vitamin D deficient mice received an much higher dose of $1,25(OH)_2D$ (2 ng/g BW, 6-h) than our vitamin D replete mice (0.5 ng/g BW, 4-h), they are expected to respond more strongly by having more DEG in their duodenum. Altogether, these conditions lead to more number of $1,25(OH)_2D$-regulated DEG in their study than ours. Hence, it is expected that while there are some overlapping between two lists, they will not be the same. Lastly, we also excluded the possibly of pre-existing age-related difference on basal transcripts being a confounder to the effect of age on $1,25(OH)_2D$-regulated DEG.

One outstanding weakness of our study is that we cannot trust the quality of data from our mature and old male groups, therefore, we cannot test our hypothesis in male mice as we did for the female mice. To avoid making the mistake by assuming that our conclusion is generalizable, we did a preliminary examination to assess whether sex might be a significant modifier for the impact of age on $1,25(OH)_2D$ action. We found there are more $1,25(OH)_2D$-regulated DEG in middle-aged male than female. But middle-aged female seems to be more responsive to

1,25$(OH)_2$D signaling than male especially for known genes involved in Ca absorption and vitamin D metabolism (i.e. Cyp24a1 and Trpv6). For example, 1,25$(OH)_2$D-induced Trpv6 level is 3-fold higher in than in male. This is consistent with findings by Song et al. (162). They found that 4-h after a single 1,25$(OH)_2$D injection (2 ng/g BW), Trpv6 mRNA is 2-fold greater in 2-month old female than in age matched male mice. While we found 1,25$(OH)_2$D-induced level for S100g at 4-h after the injection is higher in middle-aged male mice than female, Song et al. found no difference at 4-h point for their 2-month old mice but it became higher in female than male from 6-16 h after the injection. Based on these evidence, we came to a cautious conclusion that it's likely our observations made using female data are accurate but we have good reason to think that we might not get exactly the same result in males. This means more work needs to be done through future research.

In conclusion, our discovery has significant meaning to the literature because we are the first to identify the molecular foundation for the intestinal resistance to 1,25$(OH)_2$D with aging, which has always been assumed as a universal phenomenon associated with low intestinal VDR level. Opposite to what people generally believe, we found the effect of aging on 1,25$(OH)_2$D-regulated gene expression varies across genes, suggesting there is no universal age-associated resistance to 1,25$(OH)_2$D action across the genome. Furthermore, we found no change of Vdr mRNA level with age. The exact mechanisms that cause this age-related resistance to 1,25$(OH)_2$D for some genes but not for others require further investigation through future research. Although our findings were based on female mice only, several precautions were taken to ensure our data is accurate, therefore, on which the conclusions were based are also correct. Furthermore, we examined whether sex affects the impact of age on 1,25$(OH)_2$D action by comparing middle-aged male versus female mice. We found there are more 1,25$(OH)_2$D-regulated DEG in male than female. However, while the fold induction of expression for some genes (e.g., S100g) is higher in male, others (e.g., Cyp24a1, Trpv6) is higher in female. These sex-related differences in the responses to 1,25$(OH)_2$D indicate our findings is specific for female mice, and it is important to conduct the experiment on male mice through future research.

4.6 Figures and Tables

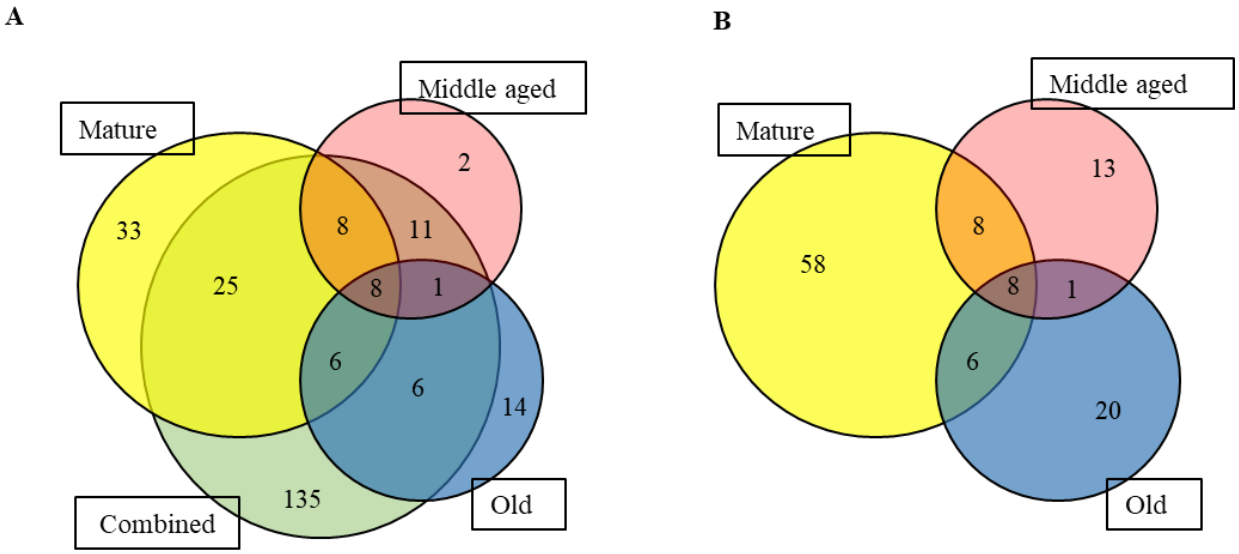

Figure 4.1. Identification of Differentially Expressed Genes (DEG) by $1,25(OH)_2D$ in the Duodenum of Female Mice.

4-month-old (mature), 11.5-month-old (middle-aged), and 20.5-month-old (old) received $1,25(OH)_2D$ (i.p. 0.5 ng/g BW) or vehicle (n=5/age/treatment). Mucosal scraping from the duodenum was collected 4 hours after the injection. Genes were considered differentially expressed at adjusted p value ≤ 0.1. Each age DEG list was compared with (A) or without (B) the combined DEG that includes all females.

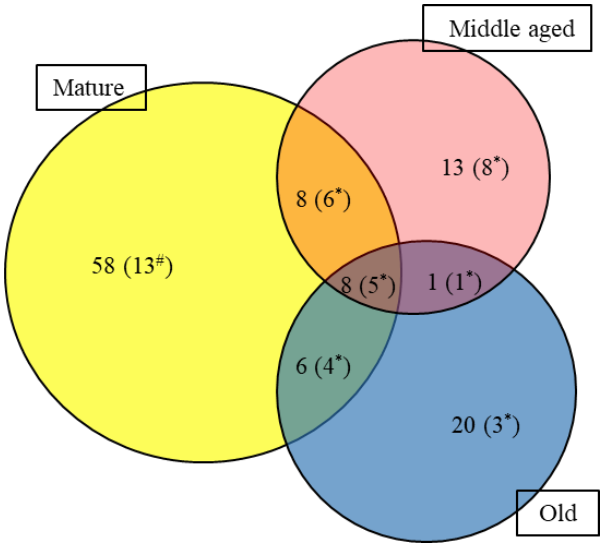

Figure 4.2. Distribution of Common Genes Shared by 1,25(OH)$_2$D-regulated Differentially Expressed Genes (DEG) in the Current Study and Known Profiling.

4-month-old (mature), 11.5-month-old (middle-aged), and 20.5-month-old (old) female mice received 1,25(OH)$_2$D (i.p. 0.5 ng/g BW) or vehicle (n=5/age/treatment). Mucosal scraping from the duodenum was collected 4 hours after the injection. Genes were considered differentially expressed at adjusted p value ≤ 0.1. The DEG list of the current study was compared to published data by Lee et al. (79) and the number of shared genes are shown as the number in the bracket. *All are also upregulated in both studies. #10 of the 13 shared genes are upregulated in both studies.

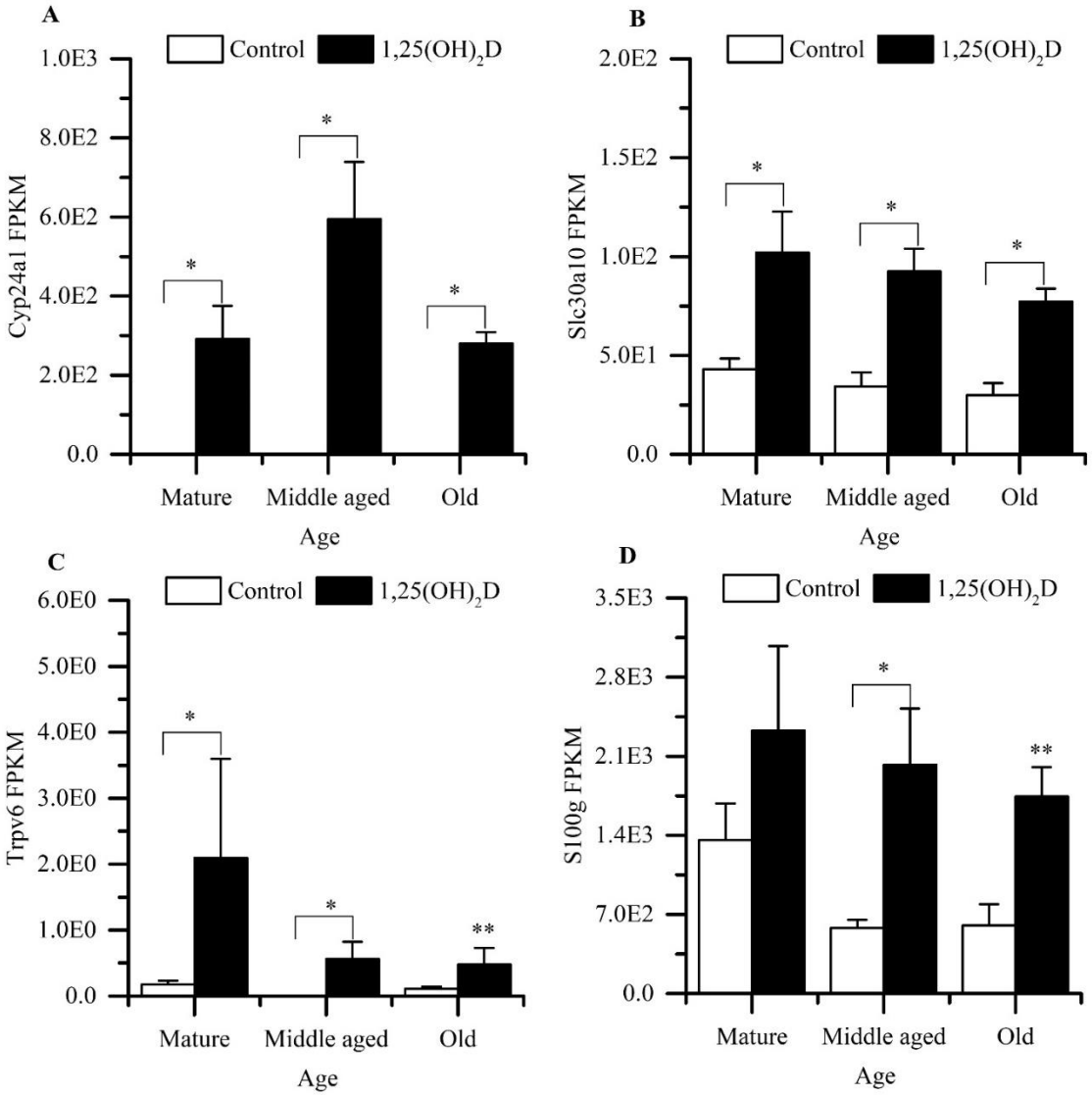

Figure 4.3. 1,25(OH)$_2$D target gene expression in duodenum of female mice by age.

4-month-old (mature), 11.5-month-old (middle-aged), and 20.5-month-old (old) female mice received 1,25(OH)$_2$D (i.p. 0.5 ng/g BW) or vehicle (n=5/age/treatment). Mucosal scraping from the duodenum was collected 4 hours after the injection. RNA was isolated from the duodenum and examined for Cyp24a1 (A), Slc30a10 (B), Trpv6 (C), and S100g gene expression by RNA-seq. Data are expressed as the mean of FPKM ± SEM (n=5 per group). *Adjusted p value ≤ 0.1 vs vehicle treated control mice of the same age. **Unadjusted p value ≤ 0.1 vs vehicle treated control mice of the same age.

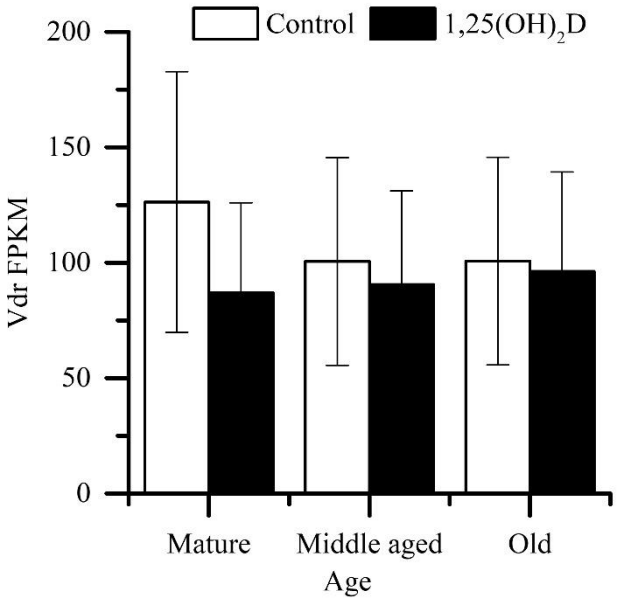

Figure 4.4. Vdr mRNA expression in the duodenum of female mice by age.

4-month-old (mature), 11.5-month-old (middle-aged), and 20.5-month-old (old) female mice received $1,25(OH)_2D$ (i.p. 0.5 ng/g BW) or vehicle (n=5/age/treatment). Mucosal scraping from the duodenum was collected 4 hours after the injection. RNA was isolated from the duodenum and examined for Vdr gene expression by RNA-seq. Data are expressed as the mean of FPKM ± SEM (n=5 per group).

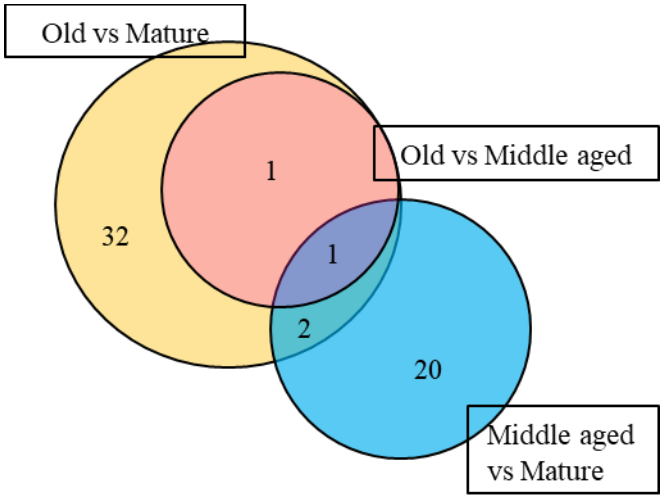

Figure 4.5. Identification of Differentially Expressed Genes (DEG) in the Duodenum of vehicle-treated Female Mice by Age.

4-month-old (mature), 11.5-month-old (middle-aged), and 20.5-month-old (old) female mice received vehicle (n=5/age). Mucosal scraping from the duodenum was collected 4 hours after the injection. Genes were considered differentially expressed at adjusted p value ≤ 0.1.

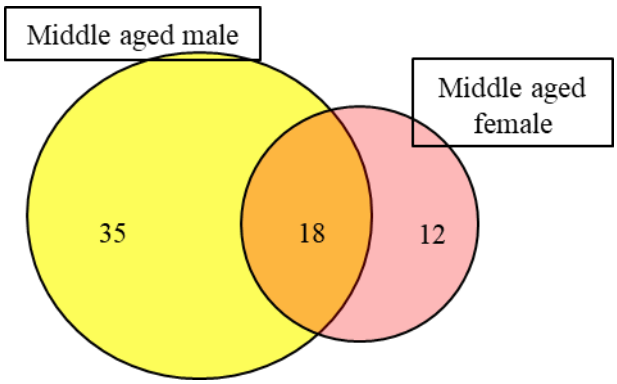

Figure 4.6. Identification of 1,25$(OH)_2$D-regulated Differentially Expressed Genes (DEG) Between Middle-aged Male and Female Mice.

11.5-month old (middle-aged) male and female mice received 1,25$(OH)_2$D (i.p. 0.5 ng/g BW) or vehicle (n=5/age/treatment). Mucosal scraping from the duodenum was collected 4 hours after the injection. Genes were considered differentially expressed at adjusted p value ≤ 0.1.

Supplemental Figure 4.7. The Hierarchical Clustering of Top 205 Differentially Expressed Genes in Response to $1,25(OH)_2D$.

4-month-old (mature), 11.5-month-old (middle-aged), and 20.5-month-old (old) male and female mice received $1,25(OH)_2D$ (i.p. 0.5 ng/g BW) or vehicle (n=5/age/treatment). Mucosal scraping from the duodenum was collected 4 hours after the injection. Genes were considered differentially expressed at adjusted p value ≤ 0.1.

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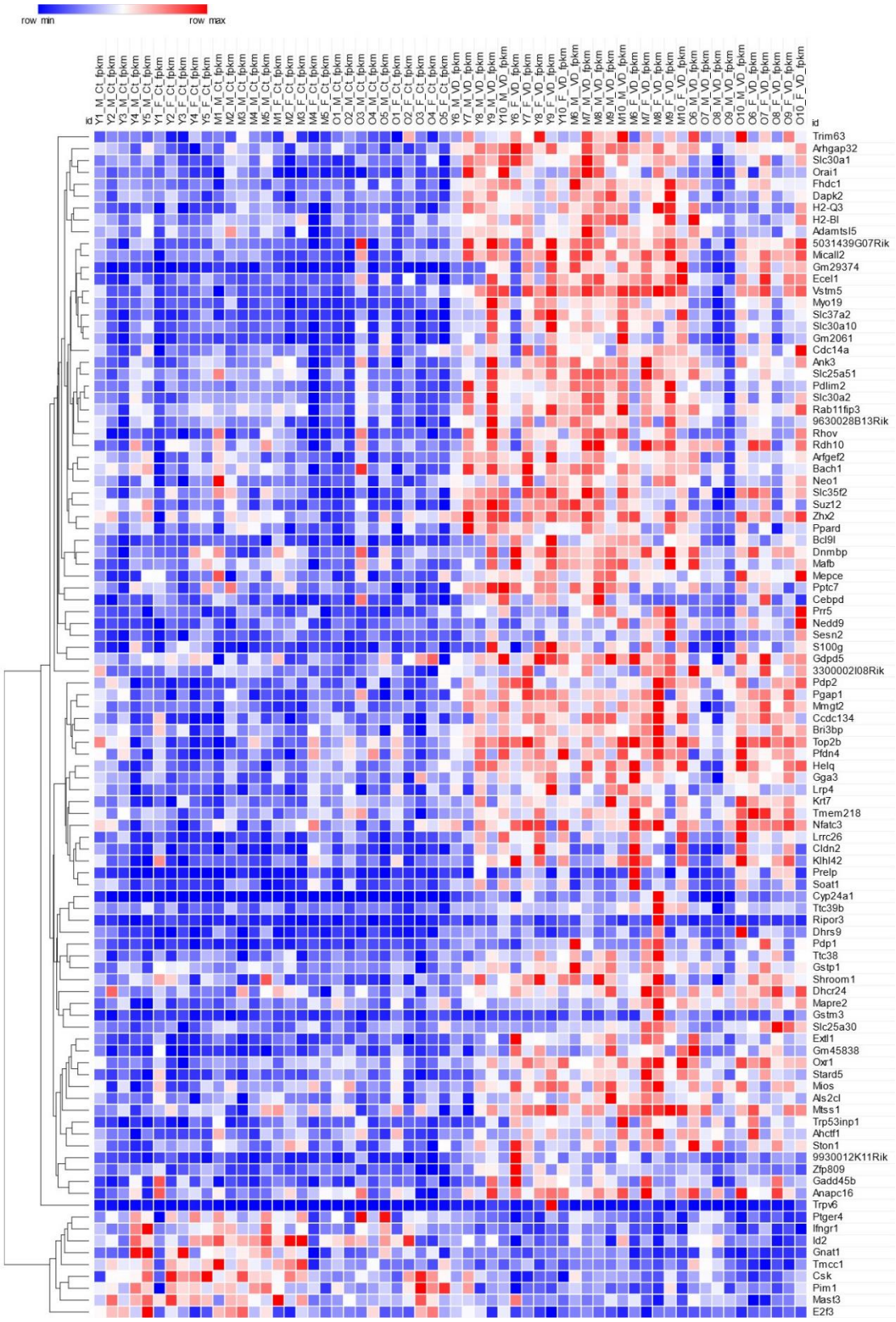
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Micall2
Gm29374
Ecel1
Vstm5
Myo19
Slc37a2
Slc30a10
Gm2061
Cdc14a
Ank3
Slc25a51
Pdlim2
Slc30a2
Rab11fip3
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Rhov
Rdh10
Arfgef2
Bach1
Neo1
Slc35f2
Suz12
Zhx2
Ppard
Bcl9l
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Mepce
Pptc7
Cebpd
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Nedd9
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Pdp2
Pgap1
Mmgt2
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Bri3bp
Top2b
Pfdn4
Helq
Gga3
Lrp4
Krt7
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Nfatc3
Lrrc26
Cldn2
Klhl42
Prelp
Soat1
Cyp24a1
Ttc39b
Ripor3
Dhrs9
Pdp1
Ttc38
Gstp1
Shroom1
Dhcr24
Mapre2
Gstm3
Slc25a30
Extl1
Gm45838
Oxr1
Stard5
Mios
Als2cl
Mtss1
Trp53inp1
Ahctf1
Ston1
9930012K11Rik
Zfp809
Gadd45b
Anapc16
Trpv6
Ptger4
Ifngr1
Id2
Gnat1
Tmcc1
Csk
Pim1
Mast3
E2f3

Table 4.1. Number of differentially expressed genes (DEG) regulated by $1,25(OH)_2D$ in the duodenum of female mice by age.

4-month-old (mature), 11.5-month-old (middle-aged), and 20.5-month-old (old) female mice were received $1,25(OH)_2D$ (i.p. 0.5 ng/g BW) or vehicle (n=5/age/treatment). Mucosal scraping from the duodenum was collected 4 hours after the injection. Genes were considered differentially expressed at adjusted p value ≤ 0.1.

Age	**n**	**$1,25(OH)_2D$-regualted DEG**				**Compare to the combined DEG list**			
		Total	Up	Down	% Up	# In common	Up	Down	% Up
Combined	15	200	129	71	64.5				
Mature	5	80	56	24	70	47	41	6	87.2
Middle-aged	5	30	29	1	96.7	28	28	0	100
Old	5	35	31	4	88.6	21	19	2	90.5

Table 4.2. Comparison of 1,25$(OH)_2$D-regulated Differentially Expressed Genes (DEG) List at Different Significance Threshold.

4-month-old (mature), 11.5-month-old (middle-aged), and 20.5-month-old (old) female mice received 1,25$(OH)_2$D (i.p. 0.5 ng/g BW) or vehicle (n=5/age/treatment). Mucosal scraping from the duodenum was collected 4 hours after the injection. For each DEG list, genes were considered differentially expressed at adjusted p value ≤ 0.1 ≤ 0.15, or ≤ 0.2, respectively.

Age	**DEG (adjusted p ≤ 0.1)**				**DEG (adjusted p ≤ 0.15)**				**DEG (adjusted p ≤ 0.2)**			
	Total	Up	Down	% Up	Total	Up	Down	% Up	Total	Up	Down	% Up
Mature	80	56	24	70	80	56	24	70	84	60	24	71.4
Middle-aged	30	29	1	96.7	31	30	1	96.8	33	32	1	96.97
Old	35	31	4	88.6	37	33	4	89.2	38	34	4	89.5

Table 4.3. Comparison of Differentially Expressed Genes (DEG) by $1,25(OH)_2D$ in the Current Study Versus Known Profiling.

4-month-old (mature), 11.5-month-old (middle-aged), and 20.5-month-old (old) female mice received $1,25(OH)_2D$ (i.p. 0.5 ng/g BW) or vehicle (n=5/age/treatment). Mucosal scraping from the duodenum was collected 4 hours after the injection. Genes were considered differentially expressed at adjusted p value ≤ 0.1. The DEG list of the current study was compared to published data by Lee et al. (79)

Age	**n**	**$1,25(OH)_2D$-regualted DEG**				**Compare to the Lee DEG list**			
		Total	Up	Down	% Up	# In common	Up	Down	% Up
Mature	5	80	56	24	70	28	25	6	89.3
Middle-aged	5	30	29	1	96.7	20	20	0	100
Old	5	35	31	4	88.6	13	13	0	100

Table 4.4. Comparison of 1,25$(OH)_2$D-regulated Gene Expression by Age among Female Mice.

4-month-old (mature), 11.5-month-old (middle-aged), and 20.5-month-old (old) female mice received 1,25$(OH)_2$D (i.p. 0.5 ng/g BW) or vehicle (n=5/age/treatment). Mucosal scraping from the duodenum was collected 4 hours after the injection. RNA was isolated and subjected to RNA-seq. Data are expressed as the mean of FPKM ± SEM (n=5 per group). [1]Differentially expressed in all three age groups. [2]Differentially expressed in Lee. *Adjusted p value ≤ 0.1 vs vehicle treated control mice of the same age. **Unadjusted p value ≤ 0.1 vs vehicle treated control mice of the same age.

Gene Symbol	Mature			Middle-aged			Old		
	Control	1,25$(OH)_2$D	Fold Change	Control	1,25$(OH)_2$D	Fold Change	Control	1,25$(OH)_2$D	Fold Change
Cyp24a1[1,2]	0.1 ± 0.09	291.93 ± 83.38*	2834	0.23 ± 0.17	594.9 ± 144.24*	2536	0.15 ± 0.05	281.04 ± 28.28*	1921
Slc30a10[1,2]	43.12 ± 5.32	101.95 ± 20.78*	2.36	34.32 ± 7.03	92.44 ± 11.44*	2.69	29.99 ± 6.07	77.15 ± 6.65*	2.54
Trpv6	0.17 ± 0.06	2.1 ± 1.5*	12.11	0.01 ± 0.004	0.562 ± 0.262*	99.38	0.11 ± 0.03	0.48 ± 0.25**	4.21
S100g	1357.45 ± 323.49	2326.64 ± 747.97	1.71	579.66 ± 73.36	2025.056 ± 495.764*	3.50	603.28 ± 185.61	1743.64 ± 258.05**	2.86
Extl1[1,2]	1.98 ± 0.54	5.57 ± 0.9*	2.80	2.08 ± 0.32	5.41 ± 0.84*	2.60	1.82 ± 0.28	4.1 ± 0.36*	2.24
Pgap1[1,2]	1.01 ± 0.19	2.85 ± 0.2*	2.83	1.06 ± 0.13	2.53 ± 0.45*	2.37	0.98 ± 0.14	2.62 ± 0.25*	2.64
Vstm5[1]	9.86 ± 1.27	26.44 ± 4.9*	2.68	8.84 ± 0.76	34.05 ± 1.29*	3.84	13.14 ± 1.76	27.39 ± 3.24*	2.07
Pdp1[1]	0.33 ± 0.06	0.87 ± 0.13*	2.61	0.37 ± 0.04	1.3 ± 0.3*	3.52	0.54 ± 0.07	1.3 ± 0.23*	2.39
5031439G07Rik[1,2]	4.42 ± 0.13	8.78 ± 1.24*	1.98	4.23 ± 0.39	9.34 ± 0.52*	2.21	4.61 ± 0.3	8.87 ± 0.67*	1.91
Gm29374[1]	0.13 ± 0.05	2.21 ± 0.58*	16.86	0.2 ± 0.08	2.94 ± 0.37*	15.14	0.08 ± 0.05	2.55 ± 0.26*	28.14

Table 4.5. The Effect of Age on Basal Differentially Expressed Genes (DEG) in the Duodenum of Female Mice.

4-month-old (mature), 11.5-month-old (middle-aged), and 20.5-month-old (old) female mice received vehicle (n=5/age). Mucosal scraping from the duodenum was collected 4 hours after the injection. Genes were considered differentially expressed at adjusted p value ≤ 0.1.

Comparison	**n**	**Total**	**Up**	**Down**
Old vs Mature	5	36	26	10
Old vs Middle-aged	5	2	1	1
Middle-aged vs Mature	5	23	13	10

Table 4.6. Comparison of Basal Differentially Expressed Genes (DEG) Between Middle-aged Male and Female Mice.

4-month-old (mature), 11.5-month-old (middle-aged), and 20.5-month-old (old) female mice received vehicle (n=5/age). Mucosal scraping from the duodenum was collected 4 hours after the injection. Genes were considered differentially expressed at adjusted p value ≤ 0.1.

Comparison	**n**	**Total**	**Up**	**Down**
Male vs Female	5	72	55	17

Table 4.7. Comparison of 1,25$(OH)_2$D-regulated Differentially Expressed Genes (DEG) Between Middle-aged Male and Female Mice.

11.5-mo-old (middle-aged) male and female mice received 1,25$(OH)_2$D (i.p. 0.5 ng/g BW) or vehicle (n=5/age/treatment). Mucosal scraping from the duodenum was collected 4 hours after the injection. Genes were considered differentially expressed at adjusted p value ≤ 0.1.

Gender	**Comparison**	**n**	**Total**	**Up**	**Down**	**% Up**
Male	1,25$(OH)_2$D vs Control	5	53	42	11	79.2
Female	1,25$(OH)_2$D vs Control	5	30	29	1	96.7

Table 4.8. Comparison of the Expression of 4 Known $1,25(OH)_2D$ Target Genes in Middle-aged Male versus Female Mice.

11.5-month old (middle-aged) male and female mice received $1,25(OH)_2D$ (i.p. 0.5 ng/g BW) or vehicle (n=5/age/treatment). Mucosal scraping from the duodenum was collected 4 hours after the injection. RNA was isolated and subjected to RNA-seq. Data are expressed as the mean of FPKM ± SEM (n=5 per group). *Adjusted p value ≤ 0.1 vs vehicle treated control mice of the same age and gender.

Gene Symbol	Male			Female		
	Control	$1,25(OH)_2D$	Fold Change	Control	$1,25(OH)_2D$	Fold Change
Cyp24a1	0.19 ± 0.11	344.32 ± 47.6*	1846.01	0.23 ± 0.17	594.9 ± 144.24*	2536
Slc30a10	47.61 ± 4.09	117.33 ± 10.82*	2.46	34.32 ± 7.03	92.44 ± 11.44*	2.69
Trpv6	0.02 ± 0.00	0.18 ± 0.07*	11.62	0.01 ± 0.004	0.562 ± 0.262*	99.38
S100g	306.46 ± 85.71	2119.71 ± 275.14*	6.90	579.66 ± 73.36	2025.056 ± 495.764*	3.50

Table 4.9. Comparison of the Expression of 1,25(OH)$_2$D Target Genes in Middle-aged Male versus Female Mice.

11.5-month old (middle-aged) male and female mice received 1,25(OH)$_2$D (i.p. 0.5 ng/g BW) or vehicle (n=5/age/treatment). Mucosal scraping from the duodenum was collected 4 hours after the injection. RNA was isolated and subjected to RNA-seq. These genes represent the 14 novel genes that were significantly vitamin D regulated in both sexes. Data are expressed as the mean of FPKM ± SEM (n=5 per group). *Adjusted p value ≤ 0.1 vs vehicle treated control mice of the same age and gender.

Gene Symbol	Male			Female		
	Control	1,25(OH)$_2$D	Fold Change	Control	1,25(OH)$_2$D	Fold Change
Extl1	2.02 ± 0.33	5.00 ± 0.66*	2.48	2.08 ± 0.32	5.41 ± 0.84*	2.60
5031439G07Rik	5.51 ± 0.27	9.57 ± 0.53*	1.74	4.23 ± 0.39	9.34 ± 0.52*	2.21
Vstm5	8.78 ± 1.53	31.71 ± 1.65*	3.61	8.84 ± 0.76	34.05 ± 1.29*	3.84
Pdp1	0.32 ± 0.07	1.2 ± 0.32*	3.74	0.37 ± 0.04	1.3 ± 0.3*	3.52
Gm29374	0.41 ± 0.15	2.73 ± 0.34*	6.69	0.2 ± 0.08	2.94 ± 0.37*	15.14
Slc37a2	14.1 ± 1.9	56.34 ± 5.46*	3.99	12.6 ± 1.9	56.99 ± 8.71*	4.52
Gm2061	9.69 ± 0.74	32.23 ± 5.87*	3.32	6.76 ± 1.69	25.89 ± 4.36*	3.83
Ecel1	1.28 ± 0.08	3.43 ± 0.29*	2.68	1.36 ± 0.11	3.89 ± 0.31*	2.86
Stard5	3.44 ± 0.66	7.98 ± 0.53*	2.32	3.9 ± 0.54	10.07 ± 1.48*	2.58
Ccdc134	2.93 ± 0.52	5.83 ± 0.65*	1.99	2.8 ± 0.38	6.68 ± 0.55*	2.38
Slc30a1	7.37 ± 0.76	12.19 ± 1.04*	1.65	4.53 ± 0.47	10.57 ± 0.51*	2.33
Myo19	3.89 ± 0.51	7.33 ± 0.28*	1.88	2.94 ± 0.25	6.84 ± 0.4*	2.33
Micall2	8.67 ± 0.39	13.76 ± 0.92*	1.58	7.04 ± 0.18	14.09 ± 0.84*	2.00
Fhdc1	2.62 ± 0.35	5.59 ± 0.48*	2.13	2.75 ± 0.17	5.26 ± 0.47*	1.91

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CHAPTER 5. FUTURE DIRECTIONS

My research projects presented from chapter 2 through chapter 4 discussed our discoveries on the spatial and temporal complexity of intestinal $1,25(OH)_2D$ action. In chapter 2 we studied the role of $1,25(OH)_2D$/VDR signaling in adult Ca absorption and bone health from the proximal-distal axis. We found that in adult mice, $1,25(OH)_2D$/VDR signaling plays a minimal role in regulating Ca absorption and protecting bone mass when dietary Ca intake is adequate. In contrast, $1,25(OH)_2D$/VDR signaling in the whole intestine, and to a lesser extent the proximal colon, is critical to upregulate Ca absorption and protect bone when dietary Ca intake is low.. Our findings filled two gaps in the literature. The first one is we are the first to directly test the importance of $1,25(OH)_2D$/VDR signaling in Ca absorption and bone health for mature adult mice. The second gap we filled is we showed the proximal colon has the $1,25(OH)_2D$-responsive Ca absorptive capacity for mature mice. This study sets the foundation for targeting the proximal colon in our chapter 3. In this chapter, we studied whether two novel compounds, Solanum glaucophyllum (Sg) leaf, which contains glycoside forms of calcitriol (1-3), and a novel synthetic 1,3-diglucuronide form of calcitriol (1,3-diG) (4, 5) to target the proximal colon and upregulate genes involved in Ca absorption (i.e. Trpv6, S100g) in 13-week old female C57BL/6J mice. We found that when calcitriol equivalent was delivered at 2 ng/day for 2 weeks, both compounds stimulated the expression of Trpv6 in the proximal colon. At this doseSg leaf did not induce a systemic effect while 1,3-diG did. At a lower dose of 1 ng calcitriol equivalent /day, 1,3-diG was still effective at inducing gene expression in the proximal colon but it did not have a systemic effect. Our data suggest that the use of this compound will require more dose optimization. Nonetheless, the discovery that the vitamin D-responsive Ca absorption machinery in the proximal colon can be targeted by these two compounds opens up directions for further research such as testing the effectiveness of these two compounds in protecting adult bone health especially for those at risk due to compromised intestinal Ca absorption ability (6, 7).

In addition to discovering the spatial complexity of intestinal $1,25(OH)_2D$ action, we also assessed its temporal complexity in chapter 4 by conducting a RNA-seq analysis of $1,25(OH)_2D$-regulated genes in the duodenum of mature, middle-aged, and old female mice. We found that although aging alters the response to $1,25(OH)_2D$, it is not an universal effect that affects all vitamin D-regulated transcripts across the genome. Furthermore, we found no change of Vdr

mRNA level with age. However, our findings are not generalizable to both sexes because we observed different duodenal responses to 1,25$(OH)_2$D treatment between middle-aged male and female mice. Therefore, this opens up another future direction that should focus on male mice. In this chapter, I will outline a few directions for future research that was built upon the discovery we made through my dissertation chapter 2-4.

5.1 Can We Use Sg leaf to Target the Proximal Colon of Mature Mice to Improve Ca Absorption and Reduce Bone Loss?

Since we have shown in chapter 3 that both the glycoside and glucuronide form of calcitriol can release active 1,25$(OH)_2$D in the proximal colon and stimulate genes for Ca absorption. The next question is to ask whether this stimulation of Ca absorptive machinery could lead to an increase in intestinal Ca absorption and reduce bone loss in adult mice, especially when dietary Ca intake is low. However, given the fact that 1,3-diG induced systemic effect at higher dose, suggesting a risk for developing hypercalcemia. Therefore, I decided to move forward in the future directions using Sg leaf at the dose that was most effective (delivers 2 ng/day calcitriol equivalent). I propose two future research directions: a) whether Sg leaf can promote Ca absorption in the proximal colon for adult mice, b) whether the upregulated-Ca absorption can leads to reduced bone loss in the long term for adult mice.

5.1.1 Short-term experiment

The short-term goal is to test whether Sg leaf can promote Ca absorption in the proximal colon of growth stable, mature mice under both adequate and low Ca intake. We hypothesize that a) low Ca diet will enhance the Ca absorption efficiency in the duodenum and proximal colon of adult mice compared to those with adequate Ca intake; b) Sg leaf treatment will still increase Ca absorption efficiency in the proximal colon of adult mice with adequate Ca intake; c) Sg leaf will increase Ca absorption efficiency in the proximal colon of adult mice under dietary Ca restriction; d) Sg-induced increase in Ca absorption efficiency will be higher for mice with inadequate Ca than those with adequate Ca intake. To test our hypotheses, C57BL/6J mice (n=48) will be fed a modified AIN93G diet (0.5% Ca, 0.4% P, 200 IU VD_3, Research Diets, Inc., New Brunswick, NJ) from weaning. At 4 months of age, mice have reached a plateau for bone Ca accrual and they are considered growth stable, mature adults (8). At this point, mice will be randomized to a modified

AIN93M diet (0.4% P and 200 IU VD_3) with either 0.5% (adequate) Ca with or without Sg leaf, or 0.2% (low) Ca with or without Sg leaf (n=12/ diet, balanced by gender). The Sg leaf-containing diet will deliver calcitriol equivalent at 2 ng/day. After 2 weeks on the experimental diets, we will use 12 mice/diet (6M, 6F) to assess the short-term effect of diet on: Ca absorption efficiency in the duodenum and the proximal colon using *in situ* ligated loops procedure, gene expression in kidney, duodenum and proximal colon (from the region adjacent to Ca absorption test).

5.1.2 Long-term experiment

Our goal for the long-term experiment is to test whether the Sg leaf treatment reduces bone loss and protects bone microarchitecture in growth stable, mature mice under both adequate and low Ca intake. We hypothesize that a) low Ca diet will cause bone loss in adult mice compared to adequate Ca diet; b) Sg leaf will not cause bone mass gain in adult mice with adequate Ca intake; c) Sg leaf will reduce bone loss and protect bone microarchitecture for adult mice under dietary Ca restriction. To test our hypothesis, we will put C57BL/6J mice (n=48) mice on the same AIN93G diet as described in the short-term experiment since weaning to 4-month of age. Then mice will be fed the same AIN93M diet with either 0.5% (adequate) Ca with or without Sg leaf, or 0.2% (low) Ca with or without Sg leaf (n=12/ diet, balanced by gender). After 16 weeks, we will evaluate the long-term effect of diet on serum $1,25(OH)_2D$ level, gene expression in kidney, duodenum and proximal colon, bone mineral content and density, and bone microarchitecture phenotypes (e.g., bone volume/total volume, trabecular number, thickness, and spacing, cortical thickness, etc.)

5.2 Does Aging Have an Effect on $1,25(OH)_2D$ Action in the Duodenum of Male Mice?

Through this future direction, we will be extending our previous observations among female mice and testing whether there is an universal effect of aging on $1,25(OH)_2D$ action in the duodenum of male mice. Based on what we've learned from the female mice, we hypothesize that age is associated with reduction in $1,25(OH)_2D$-regulated transcripts for male but this effect is not universally present. We will use 4-month old (mature), 11.5-month old (middle-aged), and 20.5-month old (old) male C57BL/6J mice (n=5/age). Male mice will be fed a modified AIN93G diet (0.8% strontium, 0.02% Ca, 0.36% P) for 2 weeks to inhibit renal $1,25(OH)_2D$ synthesis.

Afterwards, mice were treated with vehicle or 1,25$(OH)_2$D (i.p. 0.5 ng/g BW, 4h). Because we observed previously that old male mice usually have the issue of enlarged seminal vesicle, we will inject the treatment solution using a two-people technique. One person will be restraining the mouse while the other person pinch and lift the skin and muscle layer to create enough space for intraperitoneal injection without hitting the enlarged seminal vesicle. Duodenal scraping will be harvested for RNA extraction and RNA-seq analysis.

5.3 Is There a Proximal-Distal Effect on Age-related Resistance to 1,25$(OH)_2$D Action in both Male and Female Mice?

In addition to what we have done or propose to do in the previous section, it would also be interesting to find out whether the effect of age-related resistance to 1,25$(OH)_2$D action in the duodenum would be different in the large intestine. The large intestine of the digestive system also plays a crucial role in whole body Ca homeostasis. It is particularly important for individuals at risk for bone loss due to conditions such as small intestine resection (6, 7, 9-11). Therefore, this type of research will potentially help to develop therapeutic strategies to improve the intestinal responsiveness to 1,25$(OH)_2$D and reduce bone loss for individuals at high risk. Our hypothesis will be that DEG in proximal colon will respond to 1,25$(OH)_2$D in a similar manner, and we will observe an universal age-related resistance to 1,25$(OH)_2$D in the proximal colon. The study is essentially the same as what we described for the duodenum. Please refer to the previous section for more information.

5.4 References

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