The Role of Calcitriol in Regulating Fetal Bone and Mineral Metabolism, as Elucidated Through Study of *Cyp27b1* Null Fetal Mice

By

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ABSTRACT

Does calcitriol play any role in regulating mineral metabolism or skeletal development *in utero*? Studies of Boston and Leuven vitamin D receptor (VDR) ablation models reported that *Vdr* null fetuses have normal serum minerals, parathyroid hormone (PTH), skeletal morphology and mineralization. However, *Vdr* null fetuses also have increased serum calcitriol, placental calcium transport, and placental expression of *Pthrp* and *Trpv6.* In the present study, we examined *Cyp27b1* null fetal mice, which do not make calcitriol, to determine if loss of calcitriol has the same consequences as loss of VDR. *Cyp27b1* null and WT females were mated to *Cyp27b1*$^{+/-}$ males, which generated *Cyp27b1* null and *Cyp27b1*$^{+/-}$ fetuses from *Cyp27b1* null mothers, and *Cyp27b1*$^{+/-}$ and WT fetuses from WT mothers. We confirmed that calcitriol was undetectable in *Cyp27b1* null fetuses; therefore, they truly lacked calcitriol and were a useful model to address the research question. *Cyp27b1* null fetuses had normal serum calcium, serum phosphorus, PTH, skeletal ash weight, ash mineral content, tibial length and morphology. Placental calcium transport was normal in *Cyp27b1* null fetuses, while qPCR of placental mRNA confirmed loss of *Cyp27b1* expression but no change in expression of key genes involved in placental mineral transport, including transient receptor potential cation channel subfamily V member 6 (*Trpv6)* and parathyroid hormone related protein (*Pthrp).* In summary, loss of calcitriol in *Cyp27b1* null fetuses borne of *Cyp27b1* null mothers did not significantly alter any measured parameter of mineral or bone homeostasis.

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LIST OF ABBREVIATIONS

1αOHase ……1-Alpha-Hydroxylase

$2^{\Delta CT}$……Comparative C_T Method

7-DHC……7-Dehydrocholesterol

24OHase……...……24-Hydroxylase

25OHase...……25-Hydroxylase

$25OHD_3$…….... 25-Hydroxyvitamin D_3

ANOVA……Analysis of Variance

bp……...Base Pairs

C……..Celsius

C-section……...Caesarean Section

CaSR……Calcium Sensing Receptor

CCAC……. Canadian Council on Animal Care

CDNA……Complementary Deoxyribonucleic Acid

C_T……Threshold Cycle

Cyp24a1……. …Cytochrome P450 family 24 subfamily A member 1

Cyp27b1……..Cytochrome P450 family 27 subfamily B member 1

DBP……Vitamin D Binding Protein

dH_2O……Deionized Water

DNA……. Deoxyribonucleic Acid

dNTPS……..Deoxyribose Nucleotide Triphosphates

ECF……...Extracellular Fluid

ED…….... Embryonic Day

EIA…… Enzyme Immunoassay

ELISA……Enzyme-Liked Imunnosorbent Assay

EtOH…… Ethanol

EDTA…….. Ethylenediamine Tetracetic Acid

FGF23……. Fibroblast Growth Factor 23

G……...Gauge

g…… Gram

HET……. Heterozygous

h……..Hour

HHM……. Humoral Hypercalcemia of Malignancy

HRP…… Horseradish Peroxidase

IACC……. Institutional Animal Care Committee

LC-MS/MS…….. Liquid Chromatography Tandem Mass Spectrometry

M……..... Molar

MBq……. Megabecquerels

M-CSF…….. Macrophage Colony Stimulating Factor

µl……Microliter

mL……... Milliliter

mM……... Millimolar

MSC…….. Mesenchymal Stem Cell

PHEX……...Phosphate-Regulating Neutral Endopeptidase, X-Linked

Sec……Seconds

NAPI 2A…… Sodium Phosphate Transporter 2A

NAPI 2B…… Sodium Phosphate Transporter 2B

NAPI 2C…… Sodium Phosphate Transporter 2C

nm…… Nanometer

NLS……... Nuclear Localization Signal

PCR……Polymerase Chain Reaction

pg……Picogram

Pi……... Inorganic Phosphate

pM……. Picomolar

pmol…… Picomole

PTH……. Parathyroid Hormone

PTH1R…… Parathyroid Hormone Receptor Type 1

PTHrP……. Parathyroid Hormone - Related Protein

RANKL……. Receptor Activator of Nuclear Factor Kappa B Ligand

g……...Relative Centrifugal Force

RNA……. Ribonucleic Acid

RT qPCR……...Real Time Quantitative Reverse Transcriptase - PCR

RXR ……...9-cis-Retenoic Acid Receptor

S100G……S 100 calcium binding protein G (Calbindin-D_{9k})

SD……... Standard Deviation

SE……... Standard Error

TRPV6…… Transient Receptor Vanilloid Type 6

UNG…… Uracil-DNA-Glycosylase

PDDR……. Pseudovitamin D Dependent Rickets

HVDRR……...Hereditary Vitamin D Resistant Rickets

VDR…….. Vitamin D Receptor

VDRE…… Vitamin D Response Element

V……... Volts

WT……... Wild-Type

LIST OF APPENDICES

I. INTRODUCTION

1.0 Preamble

The following thesis involves the study of fetal mice which are unable to synthesize calcitriol. In order to understand and place this thesis in context, I will begin with a background review of bone and mineral metabolism. This will be followed by a discussion of how fetal bone and mineral metabolism differs from that of the adult, as well as what is currently known about the role of calcitriol from studies of human and animal models.

Throughout this thesis the term phosphorus is used for consistency and simplicity, and because that is what is measured. It is acknowledged that in serum phosphorus predominantly exists as inorganic phosphates (dihydrogen and monohydrogen phosphate), while in bone it is largely in the form of hydroxyapatite, and in soft tissues and extracellular fluid (ECF) there are an abundance of organic phosphates complexed with carbohydrates, lipids, and proteins.

There are many references to genes and proteins throughout this thesis. Please note that the following nomenclature is used throughout this thesis: human genes are written in uppercase font, genes in reference to animal models are written in italics and proteins are written in font matching the standard text. In cases where both human and animal models are being referenced, genes are referenced using the human nomenclature.

1.1 Adult calcium and bone homeostasis

1.1.1 Structure and function of bone

Although often misconceived as an inert structure, the skeleton is actually one of the body's most metabolically active organs. As the largest organ of the human body, the skeleton makes up 15 % of the body's weight, and comprises the highly specialized internal framework of the body. Beyond providing the internal structure of the body, the skeleton plays a critical role in the protection of internal organs, providing an attachment for muscle, and hosting hematopoiesis of bone marrow.[1] Furthermore, bone is key player in mineral homeostasis, as it functions as a supply of mineral that can be rapidly mobilized.

The bone is made up of two osseous tissues: cortical (compact) bone and trabecular (cancellous) bone. The cortical bone comprises the hard, outer layer of bone[1] which forms a protective layer around the internal bone cavity. Cortical bone makes up nearly 80% of skeletal mass and is critical in providing structural support and weight bearing ability because of its high resistance to bending and torsion.[1] By contrast, trabecular bone contributes approximately 20% of the skeleton and is largely found at the ends of long bones, as well as in the pelvis, skull, ribs and vertebrae.[1] Trabecular bone is comprised of a meshwork of rigid trabeculae, thereby reducing skeletal weight without compromising bone strength. As the bone remodeling process begins at the bone surface, trabecular bone is more metabolically active than cortical bone, but also more vulnerable to damage when net bone loss occurs.[1]

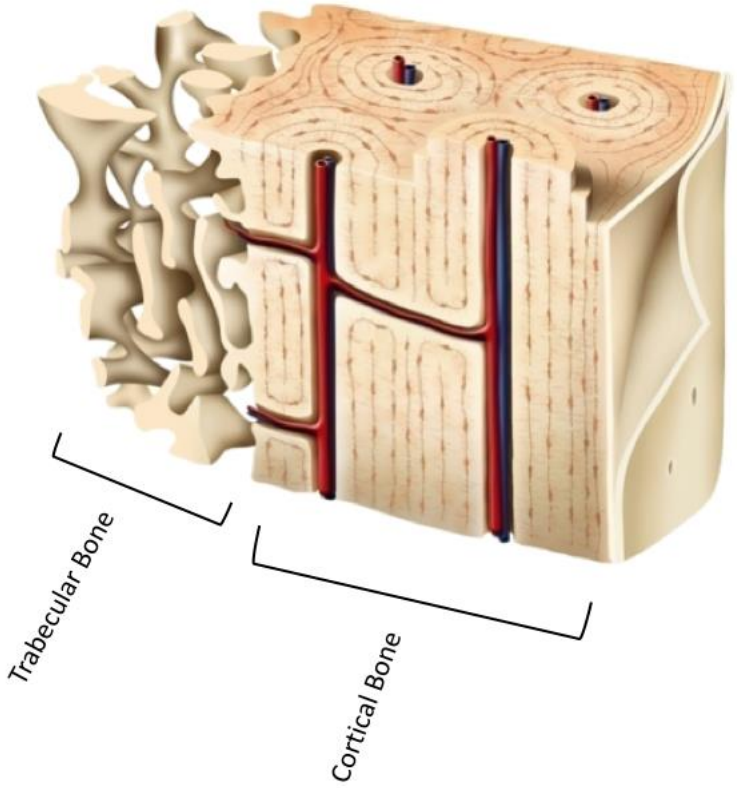

Figure 1: Cortical and Trabecular Bone. Cortical bone comprises the hard, outer layer of bone which forms a protective layer around the internal bone cavity. Trabecular bone comprised of a meshwork of rigid trabeculae, thereby reducing skeletal weight without compromising bone strength. Used with permission from Dove Medical Press © 2015 (Appendix A).

Unlike most other connective tissue matrices, bone is highly mineralized and has the unique ability to constantly regenerate itself through the process of bone turnover.[2] There are three cell types in bone: the bone-forming osteoblasts, which when embedded in the mineral matrix become osteocytes, and the osteoclasts which are the bone resorbing cells.[2]

1.1.1.1 Osteoblasts

Osteoblasts are mononucleated cells that originate from mesenchymal stem cells (MSCs), and compromise 4-5 % of total cells in bone.[3] Osteoblasts are critical in the creation and maintenance of the skeletal architecture, responsible for deposition of bone matrix and for osteoclast regulation. Osteoblasts initiate the process of bone formation by secreting osteoid, the unmineralized part of bone matrix that forms prior to maturation of bone tissue.[1] Osteoblasts then deposit mineral, in the form of calcium, into the osteoid to mineralize the bone. As bone mineralizes, some osteoblasts become trapped within the matrix, becoming osteocytes.

1.1.1.2 Osteocytes

Some osteoblasts mature into osteocytes, which have deposited a mineralized matrix surrounding themselves, as opposed to remaining on the bone surface as functional osteoblasts.[1] Osteoblasts mature into osteocytes through a process called osteocytogenesis, which involves a number of key osteogenic markers including osterix, bone sialoprotein, alkaline phosphatase, and steocalcin.[4] In the adult skeleton, osteocytes

comprise 90-95% of all of the bone cells.[4] These cells are evenly dispersed throughout the mineralized matrix and produce a dense network by connecting each other via gap junctions on their dendritic processes.[4] Although historically osteocytes were believed to be passive cells, they are more recently understood to play a critical role in the normal function of the skeleton,[3,5] particularly in targeted bone remodelling. Osteocytes are able to inhibit bone formation by way of sclerostin production. Conversely, when sclerostin production is inhibited, there is stimulation of bone formation. Furthermore, osteocytes express osteoclast specific genes and proteins which allow them to demineralize their surroundings, through an active process called osteocytic osteolysis. Later osteocytes act like osteoblasts again to restore mineral to their surroundings. [6-8]

1.1.1.3 Osteoclasts

Normal physical activity can cause wear and microcracks within the bone known as microdamage. Bone remodelling, the process by which mature bone tissue is removed from the skeleton and new bone tissue is formed, is very important in the maintenance of the skeleton's strength and ability to repair microdamage. This process is also important for the maintenance of blood mineral and alkali concentrations. In fact, when dietary absorption mineral is insufficient, the bone remodelling process accelerates in order to try to maintain sufficient mineral in the circulation. Osteoclasts are largely responsible for the bone resorption process. Osteoclasts are large, multinucleated cells which belong to the monocyte-macrophage family. These cells make up only 1-2 % of bone cells and primarily function to resorb the bone matrix.[9] Osteoclasts produce a number of enzymes, chiefly alkaline phosphatase, as well as acid, which break down collagen, calcium and

phosphorus in the bone. First, mineralized bone is broken down into fragments, then the osteoclasts engulf the fragments and digests them within cytoplasmic vacuoles. Calcium and phosphorus released from the bone during osteoclastic resorption of the bone are then released into the bloodstream to meet the body's physiological requirements.

1.1.2 Serum minerals

1.1.2.1 Calcium

Calcium is the most abundant mineral in the human body, with approximately 1000 g of calcium present in the adult. Nearly all of the body's calcium (99 %) exists in the skeleton as hydroxyapatite crystal [$(Ca_{10}(PO_4)_6(OH)_2$],[10] which provides skeletal strength and provides a dynamic store to maintain intra- and extracellular calcium concentrations. The remaining 1 % of calcium can be found in the blood, ECF and soft tissues. Of the blood total calcium, 50 % is ionized (biologically active), 40 % is bound to albumin, and 10 % exists as a complex with either citrate ($C_6H_5O_7^{3-}$) or phosphate (PO_4^-) ions.[11] As ionized calcium is the biologically active form, it can be used as a more precise measure of calcium normality. A G-protein coupled receptor, called the calcium sensing receptor (CaSR), plays a critical role in calcium homeostasis through its ability to sense free ionized calcium.[2] The CaSR is the principal mechanism for sensing calcium in the parathyroid cells, C cells, and several nephrons in the kidney, as well as in the bone and intestine.[12]

1.1.2.2 Phosphorus

Phosphorus is the second most abundant essential mineral in human body after calcium. It not only plays a role in numerous biologic processes, including energy metabolism and bone mineralization, but also provides the structural framework for deoxyribonucleic (DNA) and ribonucleic acid (RNA). A large portion of phosphorus in the body (80-90 %) exists in bone and teeth in the form of hydroxyapatite crystal, with the remainder distributed in soft tissues and ECF.[13] There are two forms of phosphorus in the ECF: organic phospholipids which are a major component of cell membranes, and inorganic phosphate, which is required for cellular function and skeletal mineralization.[14] It is believed that there is a phosphorus sensor, which acts analogous to the CaSR to regulate serum phosphorus, however, the mechanism by which phosphorus is sensed is currently unknown.

1.1.3 Calciotropic and phosphotropic hormones

Normal calcium and bone homeostasis in the adult can almost entirely be explained by the interactions of several regulatory hormones, including parathyroid hormone (PTH), fibroblast growth factor-23 (FGF23), calcitriol, PTHrP, and the sex steroids (estradiol and testosterone). Loss of any one of these hormones can have significant consequences for the adult.

1.1.3.1 PTH

PTH is produced by the chief cells of the parathyroid glands as a pre-pro-peptide consisting of 115 amino acids. Following removal of the 25 amino acid pre-sequence and the 6 amino acid pro-sequence, the mature PTH peptide, 84 amino acids in length, is secreted.[15] PTH is secreted in response to small decreases in blood ionized calcium levels, or increased phosphorus levels, in order to maintain calcium and phosphorus homeostasis in the blood. PTH aids in calcium homeostasis by promoting resorption of bone and thereby release of skeletal calcium, acting on the kidneys to reabsorb calcium, increasing renal phosphorus excretion, and enhancing intestinal calcium absorption by stimulating *Cyp27b1*, which encodes 1-alphahydroxylase (1αOHase), the enzyme necessary for the final hydroxylation step to form active calcitriol. High blood ionized calcium and calcitriol inhibit PTH release, while high serum phosphorus stimulates PTH. PTH is also regulated by FGF23, which promotes excretion of phosphorus into the urine, and also reduces circulating calcitriol levels, thereby decreasing intestinal calcium and phosphorus absorption.[16]

Blood ionized calcium levels are sensed within a tight range by the CaSR on the surface of the parathyroid cell, which is abundantly produced by the plasma membrane of these cells.[16] The CaSR becomes activated upon binding to calcium, which leads to inhibition of PTH synthesis and release.[16] PTH binds to Parathyroid Hormone Recepor Type 1 (PTH1R) on the osteoblast surface, thereby stimulating osteoblast activity in order to produce bone matrix.

Prolonged or higher amplitude stimulation by PTH causes a downregulation in osteoblastic bone-forming activity, and instead, triggers osteoblasts to produce Receptor Activator of Nuclear Factor kappa-B Ligand (RANKL). In turn, RANKL stimulates osteoclast formation, recruitment and activity.[16] In this way, PTH couples osteoblast and osteoclast activity determining which process will predominate.

1.1.3.2 FGF23

FGF23 is a hormone produced by osteocytes and osteoblasts, which acts on distant tissues to regulate the supply of phosphorus at the bone surface.[17] Study of disorders of phosphorus homeostasis has demonstrated that FGF23 plays a critical role in regulation of renal phosphorus and vitamin D metabolism. FGF23 has overlapping function with PTH to reduce phosphorus reabsorption in the kidneys; however, it has opposing effects on calcitriol homeostasis.

FGF23 acts on two main transport proteins within kidney tubules, sodium-phosphate transporters NaPi2a and NaPi2c, in order to regulate renal phosphorus. Napi2a and Napi2c are expressed in the apical membrane of the proximal tubule where they are responsible for regulating renal phosphorus reabsorption. FGF23 downregulates *Napi2a* and *Napi2c*, thereby leading to increase renal phosphorus excretion.[18]

FGF23 acts to reduce calcitriol through two ways. The first is through a reduction in renal expression and activity of 1αOHase, also known as Cytochrome P450 family 27 subfamily B member 1(*Cyp27b1*), which synthesizes calcitriol by hydroxylating 25OHD.[19] The second is through increased expression and activity of 24-hydroxylase (24OHase), through Cytochrome P450 family 24 subfamily A member 1 (*Cyp24a1*), which catabolizes calcitriol and 25OHD by adding a hydroxyl group in the 24 position.[19] Through its ability to reduce serum calcitriol, FGF23 acts indirectly to reduce intestinal phosphorus absorption. FGF23 may also act directly to decrease intestinal phosphorus absorption through interaction with intestinal sodium-phosphate transport protein NaPi2b, located on the villi of the small intestine. By way of FGF23's direct and indirect actions on intestinal phosphorus absorption, total serum phosphorus is reduced. In contrast, loss of FGF23 leads to hyperphosphatemia, due to loss of renal phosphorus excretion, as well as markedly high levels of calcitriol which lead to increased intestinal absorption of phosphorus.[19]

FGF23 also acts on the parathyroid glands to inhibit PTH, which contributes to lowering serum calcitriol.[20] High serum phosphorus and calcitriol are potent stimuli for FGF23 synthesis and release, whereas PTH modestly stimulates FGF23.[20]

1.1.3.3 Vitamin D production and metabolism

Calcitriol is the active, or hormonal, form of vitamin D, and plays numerous biologically important roles. Vitamin D is either formed in the skin from 7-dehydrocholesterol (7-

DHC) in response to UV exposure, or ingested through the diet from both food and supplements in the form of Vitamin D_3.[21] The conversion from biologically inactive Vitamin D_3 to the active form calcitriol requires two hydroxylation reactions (Figure 1). Vitamin D_3 is preferentially removed from the skin and transported into the circulation by the vitamin D binding protein (DBP).[21] DBP shuttles vitamin D to fat and muscle for storage and to the liver, where the first hydroxylation reaction occurs. Here vitamin D is metabolized to 25-hydroxyvitamin D (25OHD) via hepatic 25-hydroxylase (25OHase). DBP then transports 25OHD to the kidney where renal 1αOHase facilitates its conversion to 1,25-dihydroxyvitamin D (calcitriol). The production of calcitriol in the kidney is tightly controlled, being stimulated by PTH and inhibited by calcium, phosphate and FGF23. Furthermore, calcitriol is able to feedback to inhibit its formation and enhance its catabolism.[21]

The availiablilty of substrate, amount of enzyme, cofactor availability and enzymatic activity of Cyp24a1 all play a role in the regulation of calcitriol through 1αOHase. Cyp24a1 encodes for the enzyme 24OHase which hydroxylates calcitriol into 1,24,25-trihyroxyvitamin D (calcitroic acid) and 1,24,25-dihydroxy-26,23-lactone, which are thought to be inactive, and can be excreted from the body through bile and urine.[21]

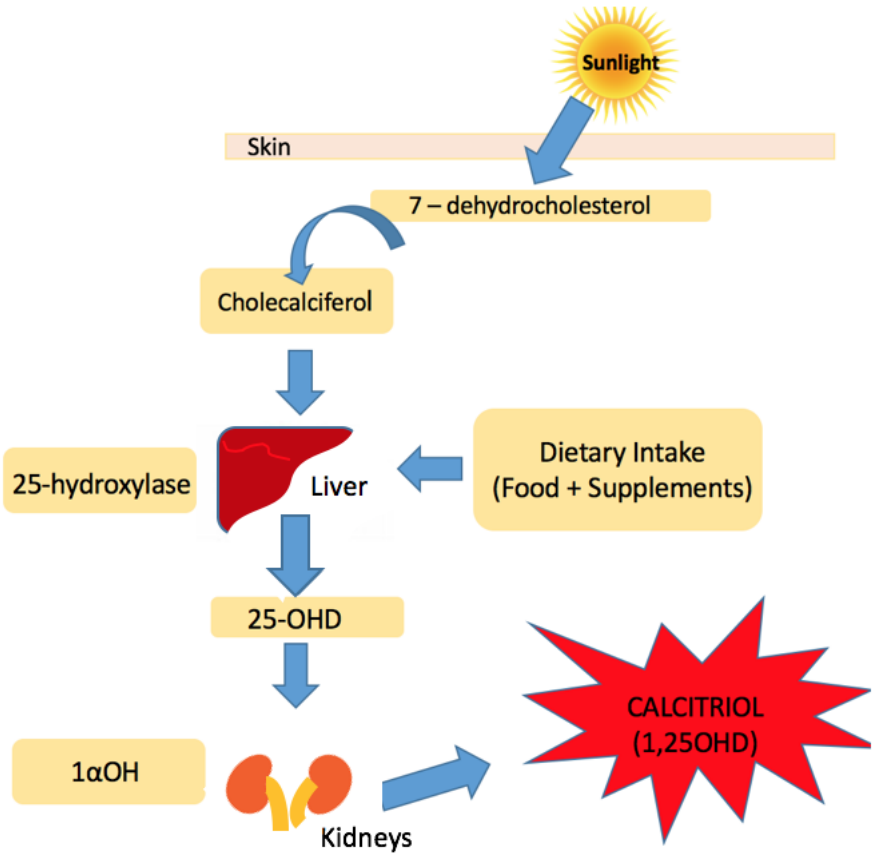

Figure 2: Vitamin D Production and Conversion into Calcitriol. Vitamin D is produced in the skin via UV exposure and ingested through the diet via food and supplements. The conversion from biologically inactive vitamin D3 to calcitriol requires two hydroxylation reactions; the first in the liver by 25OHase and the second in the kidney by 1αOHase.

1.1.3.3.1 Calcitriol

Calcitriol plays an important role in both the child and adult by increasing blood calcium and phosphorus levels. It increases the absorption of both in the intestines, increasing reabsorption of both by the kidneys, and aiding in the release of calcium and phosphorus from bone (Figure 2). Calcitriol may also play a role beyond bone and mineral homeostasis, as observational studies support an association between vitamin D and cardiovascular, immune, musculoskeletal and metabolic disorders.[22] The focus of my thesis is on the role of calcitriol in fetal bone and mineral homeostasis.

Studies of mice which lack the vitamin D receptor (*Vdr* null) have demonstrated that calcitriol's main action is to increase intestinal absorption of calcium and phosphorus.[23,24] When the demand for calcium increases, such as during growth, pregnancy, or lactation, increased synthesis of calcitriol in turn increases the efficiency of intestinal calcium absorption.[21] Calcium is absorbed in the intestines by an energy-dependent, transcellular (active) pathway, and also a paracellular (passive) pathway through tight-junctions (Figure 3).[25] Calcitriol is the main stimulator of active intestinal calcium absorption through genomic actions. Calcium enters intestinal cells through the apical calcium channel, transient receptor potential vanilloid type 6 (TRPV6),[26] and is translocated through the interior of the enterocyte, likely carried by calcium binding protein Calbindin-D_{9k} (S100G).[26] Calcium is then pumped out of the basal membrane of the cell via the intestinal plasma membrane pump Ca^{2+}-ATPase (Figure 3). There is increasing evidence to also suggest that calcitriol can also enhance passive intestinal calcium absorption by regulating tight junction proteins.[27]

The kidneys also play an important role in calcium homeostasis. When there is a drop in calcium levels in the blood, PTH acts on the kidneys to retain calcium and excrete phosphorus.[16,28] When PTH is bound to PTH1R, there is increased activity of 1αOHase leading to an increased calcitriol synthesis. In turn, the increase in calcitriol stimulates intestinal calcium absorption to increase calcium levels in the blood stream.

The skeleton serves as major supply of calcium and phosphorus when intestinal absorption and renal handling are not enough to maintain normal mineral levels within the blood. There is some amount of calcium and phosphorus that are readily available to be mobilized from bone, but these stores are rapidly depleted. When greater or prolonged release of calcium from the bone is required, osteoclastic resorption must be activated in order to supply the blood with the mineral it needs. In periods of even greater demand for calcium, osteocytic osteolysis is also activated.

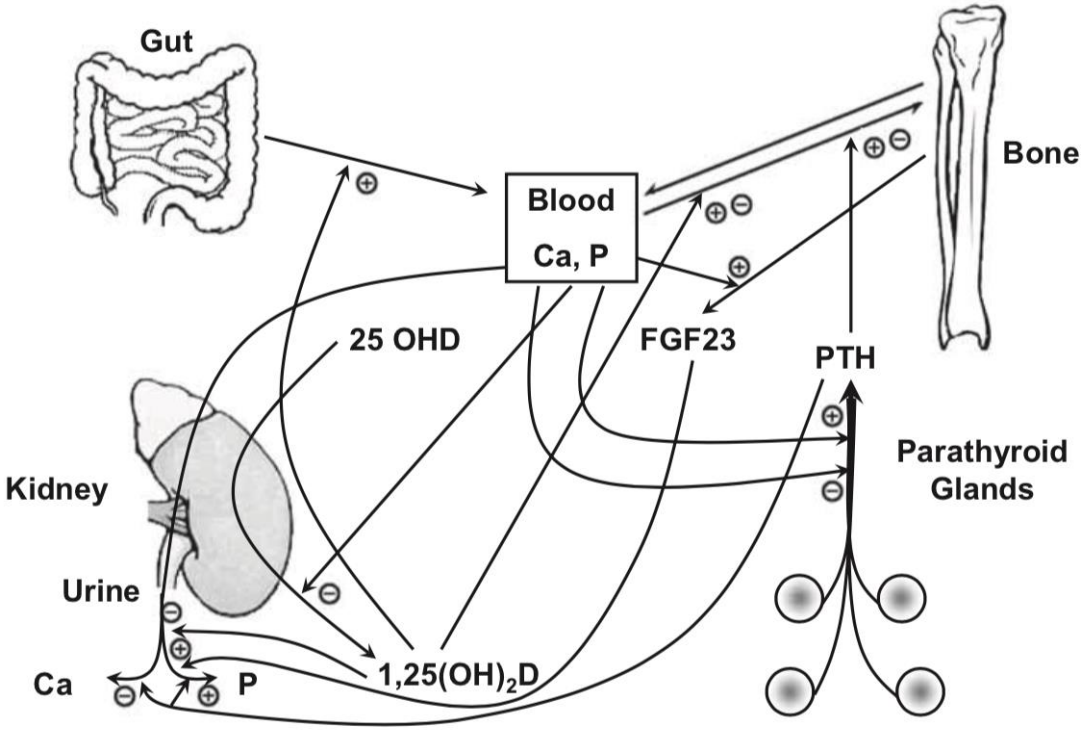

Figure 3: Classical actions of calcitriol.[21] Calcitriol interacts with PTH and FGF23 to regulate calcium and phosphorus homeostasis. Production of calcitriol is stimulated by PTH and inhibited by FGF23 in the kidney. In turn, calcitriol acts to inhibit PTH synthesis and stimulate FGF23. Used with permission from John Wiley & Sons, INC, © 2013 (Appendix B).

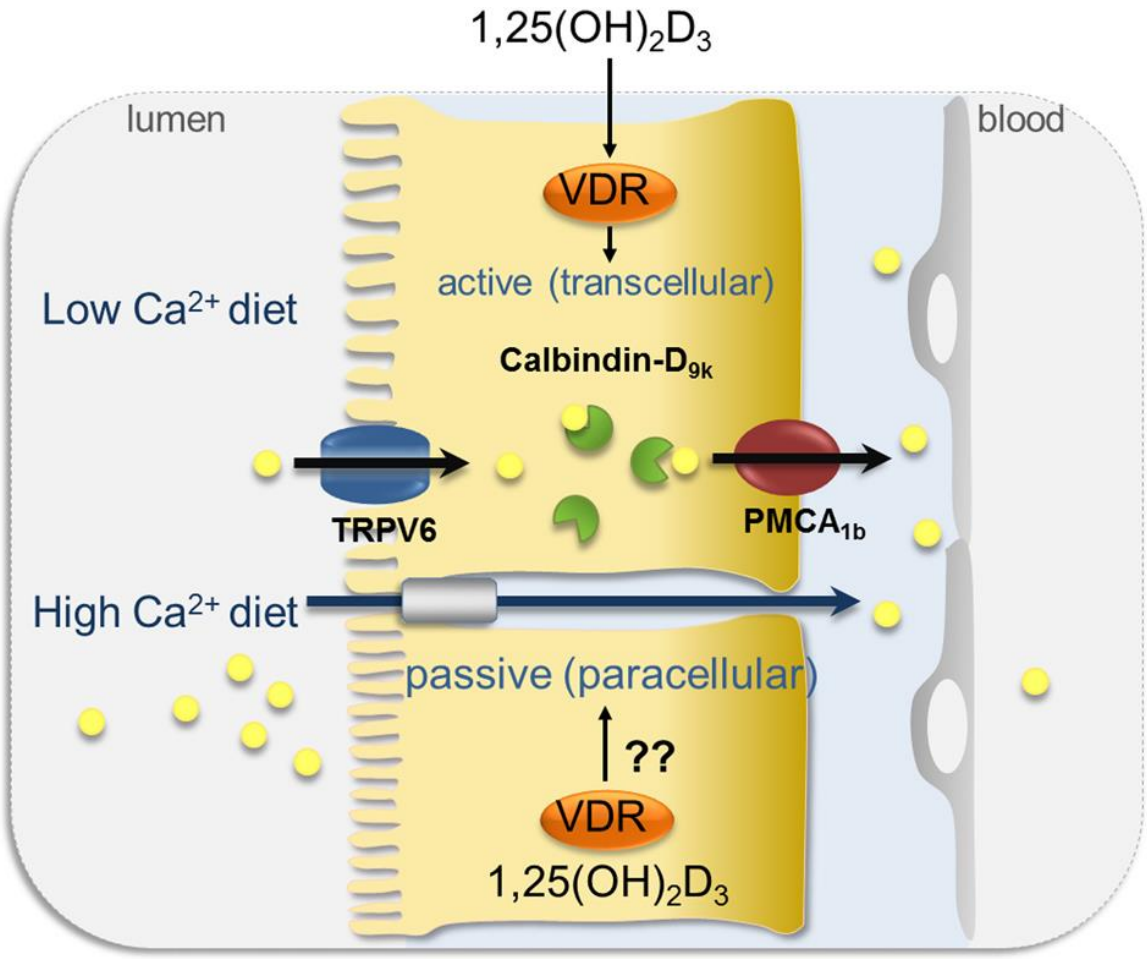

Figure 4: Active and passive intestinal calcium absorption. Calcium enters the intestine through the apical calcium channel, TRPV6, is translocated through the interior of the enterocyte, which is thought to occur with help of the calcium binding protein Calbindin-D9k. Calcium is then pumped out of the basal membrane of the cell via the intestinal plasma membrane pump Ca^{2+}-ATPase. Reprinted by permission from MacMillan Publishers Limited ©2014 (Appendix C).

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1.1.3.3.2 Vitamin D receptor

Calcitriol is transported to target cells where it diffuses into the cytoplasm and binds to the VDR. VDR possesses a nuclear localization signal (NLS) and may enter the nucleus bound to calcitriol; alternatively, calcitriol may diffuse into the nucleus and bind to the unbound VDR that has translocated to the nucleus.[29] Calcitriol binds stereospecifically to the VDR causing a conformational change into a transcriptionally active form. Once transcriptionally active, VDR is able to form a heterodimer with the 9-cis-retenoic acid receptor (RXR).[29] The VDR/RXR heterodimer recognizes a specific DNA sequence or vitamin D response element (VDRE) made up of two hexameric nucleotide half-sites separated by three base-pairs (Figure 4).[30] These actions trigger the expression of networks of target genes whose functions combine to cause tissue-specific biological responses including: complex actions required for mineral homeostasis and control of growth, differentiation and activity of numerous cell types including those of the immune system, skin, the pancreas and bone as well as many other targets.[31]

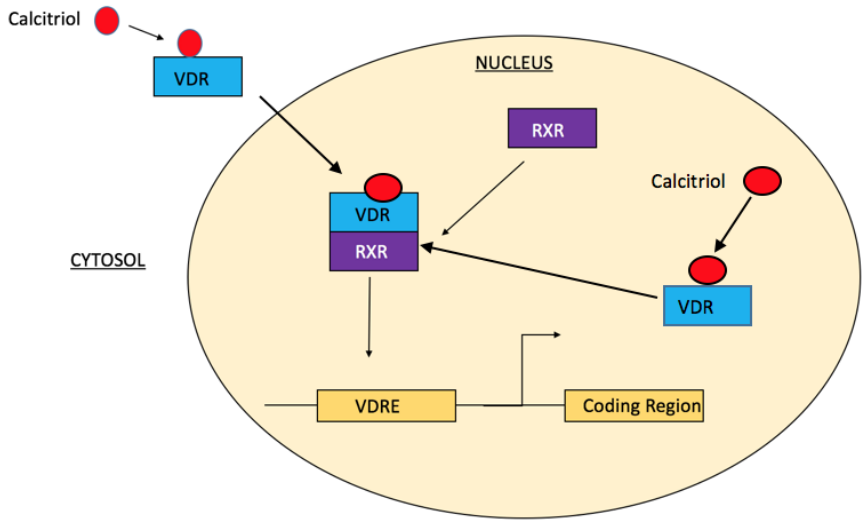

Figure 5: VDR gene structure and regulation of vitamin D dependant target genes. Calcitriol binds stereospecifically to the VDR causing a conformational change into a transcriptionally active form. Once transcriptionally active, VDR forms a heterodimer with RXR. The VDR/RXR heterodimer recognizes a specific DNA sequence, VDRE, which triggers the expression of networks of target genes whose functions combine to cause tissue-specific biological responses.

1.1.4 PTHrP

In the early 1940's, studies of humoral hypercalcemia of malignancy (HHM), characterized by an abnormal elevation of serum calcium associated with malignant tumors, theorized a "PTH-like" hormone.[32] Subsequent studies led to the biochemical characterization of HHM and the identification and characterization of PTHrP.[33,34] Both PTHrP and PTH genes share high sequence homology at the amino-terminal portion, such that the genes share 8 of the first 13 amino acids and predicted secondary structure of the subsequent 21 amino acids.[34-36] These similarities allow both peptides to bind and activate the same receptor, PTH1R.[37]

In the adult, PTHrP is typically absent from the circulation with exception to a gradual increase during pregnancy, coming from the placenta and the breasts, and a more pronounced increase during lactation. Although PTHrP doesn't normally have an endocrine function, it may have an autocrine, paracrine or intracrine function in tissues where it is locally produced.[38]

As PTHrP is expressed in almost all tissues, it serves a variety of functions in the body, including in the skeleton, the cardiovascular system, the placenta and the breast. In the skeleton, PTHrP helps to synchronize chondrocyte differentiation in the growth plates of long bones, stimulating growth.[39,40] Within the cardiovascular system, PTHrP is induced by vasoconstrictive agents and acts as a vasodilator in resistance vessels, suggesting that it may act as a local modifier of blood flow.[41] Perhaps the most well-known functions of PTHrP are within the placenta and the breast. During pregnancy, PTHrP is critical in

active transport of calcium from the mother to the fetus.[42] PTHrP remains critical into lactation where it is involved in the regulation of calcium metabolism and has been found to be highly expressed in the milk.[43,44]

1.1.5 Gonadal steroid hormones

Sex is an important determining factor in the size and shape of the skeleton, with the differences in skeletal shape between sexes reflecting the need of the female skeleton to accommodate gestation and delivery of offspring. Estradiol is essential for the bone changes that occur during puberty in both males and females. Females lacking aromatase, an enzyme in the steroid synthesis pathway necessary for synthesis of estradiol, and males lacking the estrogen receptor, do not undergo a growth spurt and do not experience epiphyseal closure.[45] During adulthood estradiol, and indirectly testosterone through conversion to estradiol, influences the growth and maintenance of the skeleton. Throughout all stages of life bone is periodically resorbed and replaced with new bone. Deficiency of estradiol causes bone loss that is associated with an unbalanced increase in the number and activity of osteoclasts and osteoblasts.[46] Conversely, sufficiency of estradiol also acts to decrease bone resorption and maintain a balance between bone resorption and formation processes. As a result of the aging process, both men and women lose bone and are prone to osteoporosis in later years. Women are more likely to develop osteoporosis as they gain less bone during puberty and experience an abrupt loss of estrogens during menopause.[45]

1.1.6 Role of the intestines

Although bone resorption can maintain serum mineral within the normal range, the only way that mineral stores in the bone can be replenished is through dietary intake. Once ingested, calcium and phosphorus are almost exclusively absorbed in the small intestine. Most of the active transport of calcium occurs in the duodenum and upper jejunum of the small intestine, while passive transport of calcium, and both active and passive absorption of phosphorus, can occur throughout the entire intestine.

Calcium is absorbed through the intestinal epithelium through one of two routes: 1) between the cells (paracellular pathway) or 2) through the cell (transcellular pathway). When calcium concentrations within the lumen are high, the passive paracellular pathway is the prominent route of absorption. The paracellular pathway is driven by the electrochemical gradient of the lumen and the integrity of intracellular tight junctions.[47] During vitamin D deficiency, excess intake of dietary calcium can increase serum calcium concentrations through this pathway. The active transcellular pathway is regulated by calcitriol and is the less predominant pathway as the concentration of calcium is usually higher in the lumen than inside the cell. The transcellular pathway involves entry of calcium via the apical calcium channel TRPV6, transport of calcium through the cell by calbindin-D_{9k} and extrusion of calcium through the basolateral membrane by Ca^{2+}ATPase (Pmca1).[27]

Phosphorus can also be absorbed through paracellular and transcellular pathways. Like paracellular absorption of calcium, paracellular phosphorus absorption is dependent on

the luminal electrochemical gradient and integrity of tight junctions. On the other hand, the transcellular phosphorus transport mechanism differs from that of transcellular calcium transport. Phosphorus is actively transported through the intestine via the sodium phosphate transporter NaPi2b, which is regulated by calcitriol, phosphorus intake and FGF23.[48]

1.1.7 Role of the kidneys

In the human adult, the kidney filters about 10 g of calcium per day. However, only 100-200 mg of this amount is excreted in the urine, meaning that approximately 98-99 % of the filtered calcium is reabsorbed into the circulation.[49] The majority of filtered calcium is reabsorbed in the proximal tubule, while 20 % is reabsorbed in the loop of Henle, 10 % by the distal convoluted tubule and 5 % by the collecting duct (Figure 5).[49] Much like intestinal calcium absorption, renal reabsorption of calcium can occur through either paracellular or transcellular pathways. Paracellular renal calcium transport makes up the majority of calcium transport within the kidney.[50] The mechanism of the active, transcellular pathway is proposed to be the same as the transcellular pathway in the intestine. Calcium enters the tubule via the apical calcium channel TRPV6, calcium is transported through the cell by calbindin-D_{9k} and calcium is secreted through the basolateral membrane by Ca^{2+}ATPase (Figure 6).[50]

Calcium handling in the kidney is regulated by a number of calciotropic hormones. The glomerular filtration rate and calcium reabsorption are increased by the actions of PTH and PTHrP.[10] PTH stimulates Cyp27b1 in order to hydroxylate 25OHD to become

calcitriol. In turn, the produced calcitriol acts on the intestines to increase intestinal calcium absorption. Calcitriol, estradiol and calcium intake also play a role in renal handling of calcium by modulating the transcription of calcium transporters TRPV5 and TRPV6.[51]

The kidneys also play a role in the regulation of phosphorus homeostasis. Maintenance of serum phosphorus levels is principally achieved by regulation of inorganic phosphate (Pi) reabsorption within the glomerular filtrate. A large portion of phosphorus absorption (approximately 85%) occurs in the proximal tubule. Within the tubule Pi transport is an energy dependent process that requires sodium.[49] Sodium phosphate transporters, NaPi2a and NaPi2c, are located on the apical brush border membrane of cells of the renal proximal tubule, using energy created from sodium movement down its gradient, to move Pi from the filtrate into the cell.[52] Phosphorus transport in the kidney is regulated by PTH, calcitriol and FGF23. Calcitriol acts to stimulate phosphorus reabsorption, while PTH and FGF23 inhibit phosphorus absorption in the renal proximal tubule and reduce the activity of NaPi2a and NaPi2c.

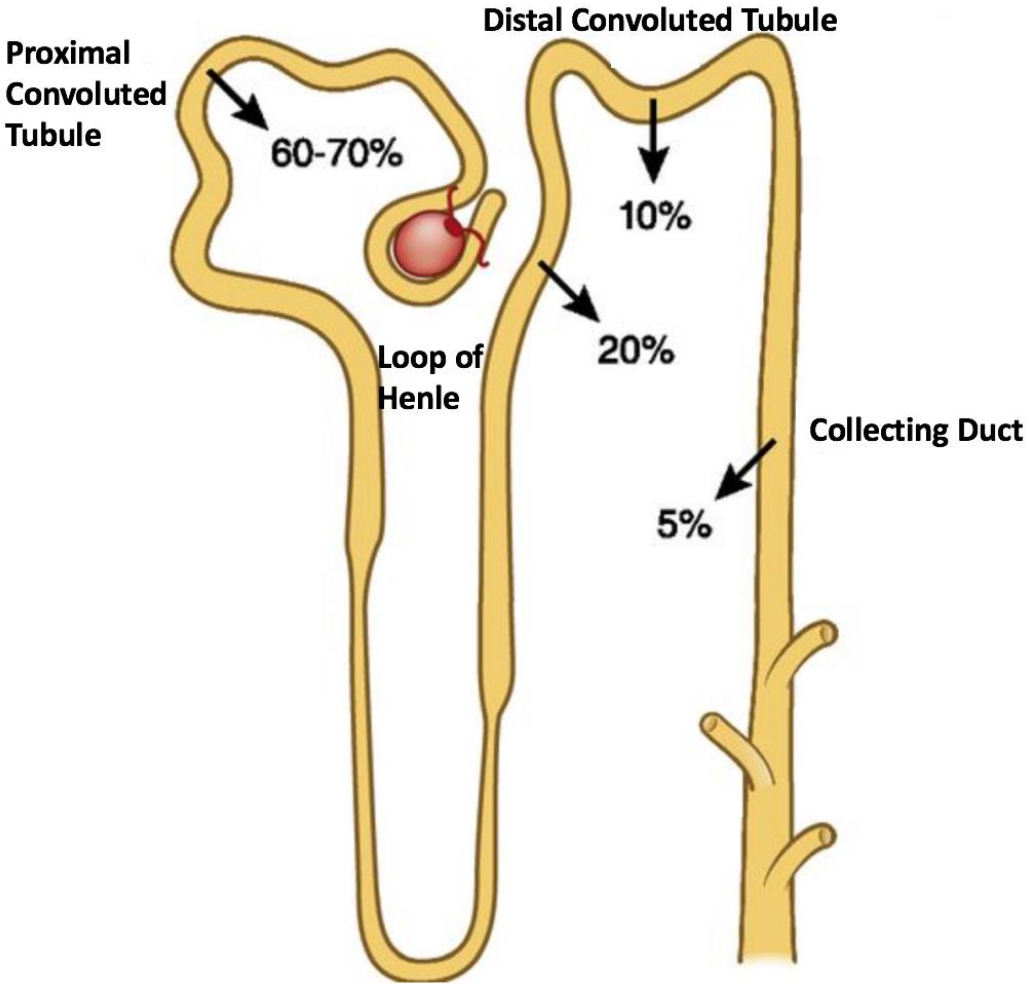

Figure 6: Renal calcium reabsorption. Calcium is filtered at the glomerulus, with the ultrafilterable portion of plasma calcium entering the proximal tubule. Within the proximal convoluted tubule, 60-70 % of filtered calcium is reabsorbed, while 10 % is reabsorbed in the distal convoluted tubule. The hoop of Henle reabsorbs about 20 %, with another 5 % of filtered calcium reabsorbed in the collecting duct. Adapted from the American Society of Nephrology © 2014 [49](Appendix D).

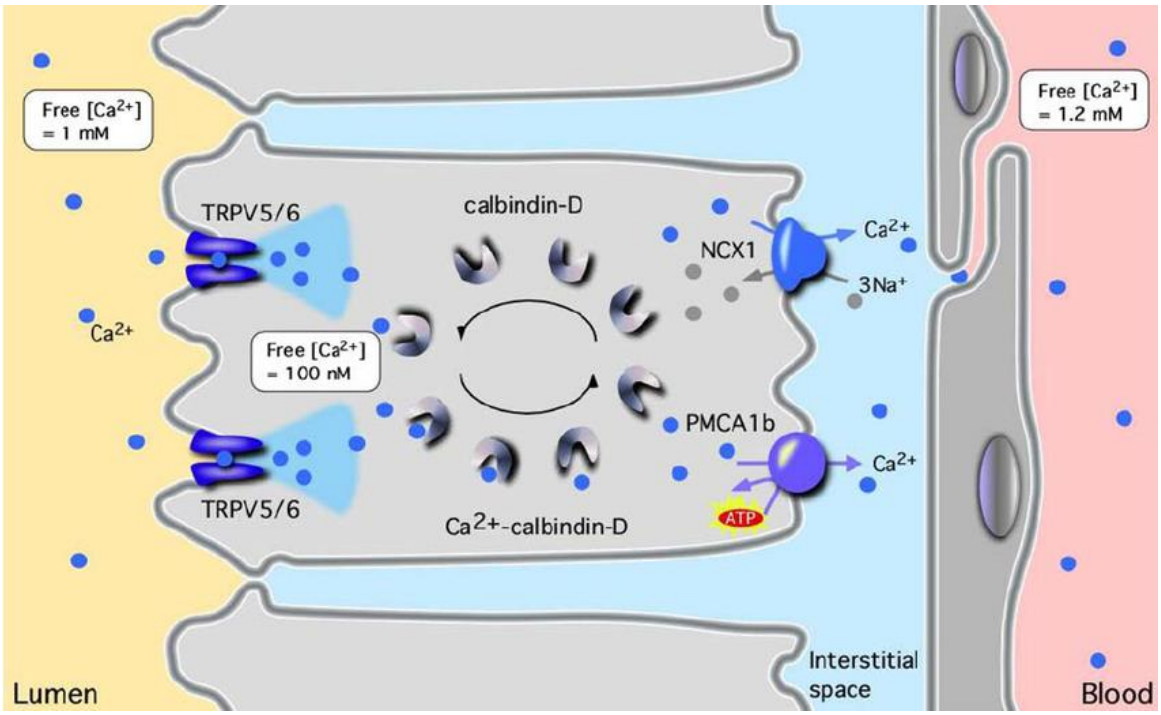

Figure 7: Renal transcellular calcium transport.[53] Calcium enters the tubule via the apical calcium channel TRPV6, calcium is transported through the cell by calbindin-D_{9k} and calcium is secreted through the basolateral membrane by PMCA1b (Ca^{2+} ATPase). Used with permission from Springer Nature © 2005 (Appendix E).

1.1.8 Role of the skeleton

Bone undergoes remodelling continuously due to the actions of osteoblasts and osteoclasts to build and breakdown bone respectively. During childhood, there is a positive bone balance, with more bone formation than resorption, in order to allow for bone growth and development. By adulthood, bone balance reaches equilibrium that is maintained through the reproductive years. However, bone resorption exceeds bone formation after menopause and in the elderly, causing a negative bone balance, which may lead to osteoporosis.

Regulation of bone remodelling occurs both locally and systemically. As previously mentioned, PTH is a key systemic regulator of bone remodelling. With physiological pulses of PTH, or pharmacological administration of short-acting PTH analogs such as teriperatide (FORTEO®), osteoblasts are stimulated causing net bone formation. In contrast, during periods of prolonged stimulation, such as during hyperparathyroidism, this leads to stimulation of net bone resorption. [54] Calcitriol regulates bone remodelling indirectly by stimulating intestinal calcium absorption, as calcium is a growth factor for osteoblasts. Furthermore, high levels of calcitriol lead to an increase in osteoclast activity, likely through the RANKL pathway.[55]

1.2 Human conditions of altered vitamin D metabolism

1.2.1 Nutritional vitamin D deficiency

Vitamin D deficiency is a global issue ranging from severe to moderate.[56-58] Severe deficiency is characterized by 25OHD levels less than 50 nmol/L (10-12 ng/mL), while

moderate vitamin D insufficiency is characterized by levels of 25OHD between 50-75 nmol/L (20-30 ng/mL).[56,59-62] Based on these criteria it has been estimated that 20-100 % of the elderly population in Canada, the United States and Europe are vitamin D deficient.[56,59,63-65] However, the elderly are not the only population at risk. Children and teenagers are also at high risk for vitamin D deficiency and insufficiency, through avoidance of sunlight and low intake of food sources that contain vitamin D.[66,67]

Calcitriol is chiefly responsible for ensuring that sufficient mineral is absorbed by the intestines to allow for skeletal growth in the child and to maintain a neutral calcium balance in the adult. Therefore, a major result of vitamin D deficiency is a reduction in intestinal calcium absorption below the level that is required to meet the demands of a growing child's skeleton.[56,59] As a consequence, mineral homeostasis is disrupted with an initial drop in ionized calcium levels and a resultant secondary hyperparathyroidism.[56,59] If the deficiency is not severe, it is possible for the elevated PTH levels to increase 1αOHase activity enough to raise calcitriol levels and increase intestinal calcium absorption to achieve normocalcemia. However, if the deficiency worsens or persists, calcitriol levels fall and hypocalcemia, hypophosphatemia and rickets ensue. This hypophosphatemia occurs in part due to reduced intestinal absorption, but is likely mainly due to secondary hyperparathyroidism which causes renal phosphate wasting.[65,68]

1.2.2 Pseudovitamin D deficiency

Pseudovitamin D deficiency (PDDR) is an autosomal recessive disorder caused by an inactivating mutation of the *Cyp27b1* gene which encodes 1αOHase, responsible from the

conversion of 25OHD into calcitriol.[69] Patients with PDDR are clinically normal at birth and most often come to medical attention at 1–2 years of age with poor growth, poor gross motor development, and generalized muscle weakness.[70] Serum chemistries in patients with PDDR are similar to, but can be more severe than, patients with nutritional vitamin D deficiency, including: hypocalcemia, hypophosphatemia and secondary hyperparathyroidism. Unlike nutritional vitamin D deficiency, serum calcitriol concentrations are usually low, however, 25OHD concentrations tend to be normal.[70] Treatment of PDDR requires physiological doses of calcitriol or 1-alpha-hydroxyvitamin D which can undergo a hydroxylation reaction in the liver to become calcitriol.

1.2.3 Hereditary vitamin D resistant rickets

Hereditary vitamin D resistant rickets (HVDRR) is a rare, autosomal recessive form of rickets characterized by resistance to calcitriol caused by a defect in the VDR gene.[71] Mutations in the VDR gene have been identified at both the calcitriol-binding and DNA-binding domains. Thus, there is a spectrum of HVDDR severity ranging from impaired binding or impaired signalling to complete absence of functional receptors. Patients with HVDRR appear normal at birth and begin to present with symptoms of calcitriol deficiency within the first 2-8 months of life, including onset of rickets, hypocalcemia, hypophosphatemia, secondary hyperparathyroidism and very high circulating concentrations of calcitriol.[72] Additionally, some patients with HVDRR develop alopecia which may be total or incomplete between ages 2 and 12 months.[72] Treatment effectiveness is dependent on the degree of hypocalcemia. If there is partial resistance to

calcitriol, treatment with pharmacologic doses of calcitriol can upregulate calcium absorption and heal the symptoms of rickets. However, if there is complete resistance to calcitriol, treatment with calcitriol is ineffective and infusion with calcium is necessary to compensate for intestinal mineral absorption.[72] Treatment is generally unable to reverse alopecia, which suggests that the skin/follicle action of calcitriol or VDR is not related to calcium and/or phosphorus metabolism. Moreover, individuals with PDDR do not get alopecia, which suggests that alopecia is a function of VDR and not calcitriol, which implies that there is another ligand for VDR.[72]

1.3 Animal models of altered vitamin D metabolism

In order to better study disorders of vitamin D metabolism, numerous animal models have been established that either lack VDR or *Cyp27b1*. These models quite closely mimic the human counterparts of PDDR and HVDRR.

1.3.1 *Vdr* null mice

The *Vdr* knock out mouse model mimics HVDRR and has been used to study the pathophysiology of altered vitamin D metabolism. There are currently several different models of VDR knockout mice, which differ by removal of either the first or second zinc finger, responsible for DNA binding. All models of *Vdr* null mice are hypocalcemic, hypophosphatemic and a characterized by secondary hyperparathyroidism and a rachitic (rickets –like) phenotype.[23,26] The rachitic phenotype of *Vdr* null mice is largely due to the indirect role of calcitriol on the skeleton through its actions to increase intestinal mineral absorption. In fact, *Vdr* null mice can be genetically rescued (phenotypically normal) by selectively expressing VDR only within intestinal cells.[73,74] On the other hand,

when VDR is ablated solely from intestinal cells or when calcium is restricted in the diet, the rachitic phenotype ensues.[74] Moreover, loss of *Vdr* from chondrocytes, osteocytes or osteoblasts of bone does not produce a rachitic phenotype, confirming that the phenotype is specific to disruption of intestinal calcium absorption.[74-76]

In order to allow for normal fertility and prevention of skeletal abnormalities, *Vdr* null mice are often kept on a "rescue diet" that is both high in calcium and phosphorus.[77] This type of diet is used in almost all studies of *Vdr* ablated mice.[78] *Vdr* null mice also experience alopecia which is unable to be cured with the rescue diet.[77] With these phenotypic similarities between *Vdr* null fetuses and babies with HVDRR, it is clear that *Vdr* null mouse is an excellent model for human HVDRR.

1.3.2 *Cyp27b1* null pigs

PDDR can be studied in the Hannover pig strain, which lack 1αOHase activity due to a naturally occurring null mutation in *Cyp27b1*.[79] In this animal model, the disease is passed on in an autosomal recessive manner. During fetal development, *Cyp27b*1 null piglets are phenotypically normal with normal serum calcium, phosphorus and skeletal mineralization.[80] By 4-6 weeks post birth, however, *Cyp27b1* null piglets begin to display a rachitic phenotype with hypocalcemia, hypophosphatemia, and low to non-detectable levels of calcitriol. It is at this stage that the pigs closely resemble the human PDDR condition.

1.3.3 *Cyp27b1* null mice

A mouse model of PDDR has also been generated to better study the disease. Mice lacking 1αOHase activity were generated by knocking out exon 8 of the *Cyp27b1* gene in order to generate a null allele.[81] Like the *Cyp27b1* null pigs, homozygous mutants are phenotypically normal at birth. However, full progression of PDDR symptoms occurs postnatally. By weaning (21 days post birth) hypocalcemia, hypophosphatemia, hyperparathyroidism and rickets are evident, and osteomalacia is present by early adulthood.[82]

1.4 Fetal bone and mineral metabolism

The fetal development period is unique in that it is a period of rapid development in which specific needs must be met by the developing fetus. Within the fetal development period, the fetus must meet several goals, including transport of mineral across the placenta, maintaining appropriate mineral concentrations within the circulation and adequately mineralizing the skeleton before birth.

Unlike in the adult, the intestines, kidneys and skeleton are not dominant sources of mineral during fetal development. Instead, the placenta must supply the fetal mineral requirements by actively transporting calcium, phosphorus, and magnesium from the maternal circulation.

The fetus must also maintain the appropriate amount of mineral in the circulation necessary for normal function of the fetal system. Among mammalian species, including

rhesus monkeys,[83,84] lambs,[85-88] calves,[87,89] rodents,[87,90] and pigs,[80,91,92] it has been consistently shown that fetal calcium concentrations are higher than that of the maternal values. The biological relevance of this increase in fetal calcium remains unknown. However, it appears to be independent of the maternal calcium level, as fetal calcium levels have been shown to be unaltered during maternal normocalcemic, hypocalcemic and hypercalcemic states.[93]

Fetal phosphorus levels also tend to be higher than maternal values, with fetal values in rodents,[94-98] lambs,[88,99,100] pigs,[80,91] calves,[87] and foals[87] observed to be 0.5-1.0 mM higher than maternal values. Just as fetal calcium levels are set independently of the mother, when mothers are hyperphosphatemic, fetal phosphorus values remain normal.[101,102] However, a few studies disagree. Studies of both pregnant rats and sheep indicate that maternal hyperphosphatemia leads to increased fetal phosphorus levels.[103,104]

Fetal serum magnesium values also appear to be set independently of maternal values; however, the data are more variable with respect to whether they are above or below maternal levels. Moderate increases in serum magnesium are observed in fetal mice and foals,[87,105,106]while fetal rats have a moderate increase to no change.[87,107] Both slight increases [88,108,109] and decreases [87,99] in serum magnesium have been observed in fetal lambs.

The fetus must also be able to meet the mineral demand necessary to successfully mineralize the fetal skeleton before birth. The majority of this mineralization process

occurs rapidly within the latter part of pregnancy. In humans, 20-30 g of calcium are necessary to mineralize the fetal skeleton, 80 % of which is accreted in the third trimester.[110] Similarly, in rats 95 % of the 12.5 g of calcium necessary for mineralization is accreted during the last 5 days of the 21-day gestation period.[111] These intervals correspond with increased rates of calcium absorption.

1.4.1 Fetal bone development

At the beginning of human fetal bone development, 8 weeks post-fertilization, the pattern of the skeleton has largely been determined. Ossification or mineralization is an important component of bone development and growth. Primary ossification centers form in the long bones and vertebrae between weeks 8 and 12 of embryo development. [112] There are two main ossification process that occur during fetal bone development: intramembranous and endochondral ossification. Intramembranous (also known as mesenchymal) ossification is the process in which mesenchymal cells in the embryonic or fibrous connective tissue become osteoblasts, in order to form the bone matrix. Conversely, endochondral ossification is the process by which cartilaginous scaffolds are created for most bones, which progressively lengthen and contribute to longitudinal growth, before being gradually replaced by bone.[113] Osteoblast progenitors in the perichondrium differentiate into osteoblasts, and as primary ossification centers expand and secondary ossification centers form, this results in the formation of the epiphyseal growth plate (Figure 7).

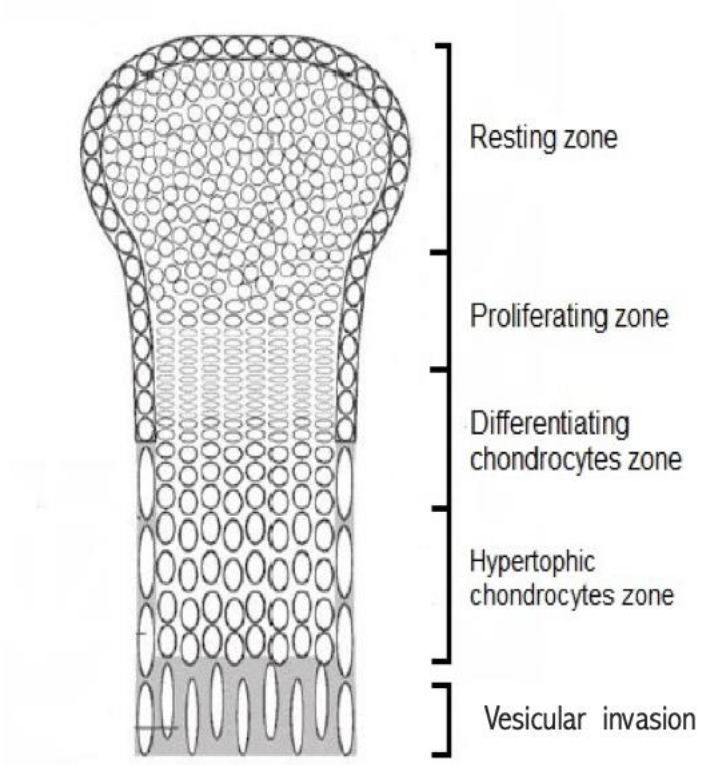

Figure 8: Endochondral bone development in the fetus. The areas of the growth plate include the resting zone, proliferating zone, differentiating chondrocytes zone and a zone of hypertrophic chondrocytes. Blood vessels invade the hypertrophic area and osteoclasts resorb the ossified cartilaginous matrix and osteoblasts derived from the bone collar replace the matrix with bone. Adapted with permission from John Wiley & Sons Inc © 2013. (Appendix F)

1.4.2 Calciotropic and phosphotropic hormones

1.4.2.1 PTH

PTH circulates at lower levels in the fetal circulation in comparison to maternal values in rodents, lambs, and calves.[89,98,114-116] Intact PTH is not able cross the placenta from the mother to the fetus; consequently, PTH within the fetal circulation is of fetal origin. PTH synthesis and release in the fetal circulation is thought to be suppressed by the activation of the CaSR on the fetal parathyroids by the high levels of total and ionized calcium.[90] Furthermore, knockout of CaSR in fetal mice leads to increased PTH.[117] Active placental transport of calcium may also contribute to the low levels of circulating fetal PTH by bringing calcium into the circulation through a route that does not require PTH.[117] Moreover, maternal hypercalcemia has been shown to increase the transfer of calcium to the fetus and further suppress the fetal PTH level, even if the fetal serum calcium does not change.[90] Low circulating PTH is not exclusive to fetal animals. In fact, infants have low circulating PTH in comparison to maternal and adult values, with concentrations as low as <0.5 picomolar (pM) observed.[118] This suppression of fetal PTH is apparent as early as 19 weeks of gestation.[119]

In mice, absence of fetal PTH causes fetal hypocalcemia, hypophosphatemia, low amniotic fluid mineral content, and reduced skeletal mineral content.[98] This suggests that, despite its low circulating levels, PTH is still required to achieve normal mineralization of the skeleton prior to birth. Despite reduced skeletal mineral content, endochondral bone development is normal, suggesting that the role of PTH in bone homeostasis is through its ability to maintain serum calcium concentration and not through osteoblast physiology.

1.4.2.2 PTHrP

Prior to the discovery of PTHrP, studies noted that infants have an increased PTH-like bioactivity and low or undetectable amounts of immunoreactive PTH in the cord blood in comparison to maternal values.[118,120]It was hypothesized that fetal blood contained a PTH-like factor that was contributing to the increased PTH-like bioactivity, which was later confirmed by the discovery of PTHrP. Human fetuses were found to have up to a fifteen-fold greater amount of PTHrP than PTH in the circulation.[121] This phenomenon was also confirmed in studies of fetal pigs[122] and sheep.[123]

Most of what is known about the role of PTHrP in fetal bone metabolism comes from the study of *Pthrp* null fetal mice. These mice display hypocalcemia, hypophosphatemia, and increased PTH.[124,125] Furthermore, *Pthrp* null fetal mice have abnormal skeletal features including: shortened limbs, domed skulls, shortened mandibles and accelerated mineralization of bone.[126] The increase in PTH suggests that it increases to compensate for lack of PTHrP; however, compensation was not achieved because hypocalcemia ensued. *Pth* null fetuses and other fetuses that lack their parathyroid glands (*Hoxa3* nulls and *Gcm2* nulls)[98] do not have a compensatory increase in PTHrP, despite being hypocalcemic. Although there is partial compensation by PTH when PTHrP is lacking, PTHrP is unable to compensate for lack of PTH. This suggests that both PTH and PTHrP play a role in fetal bone and mineral homeostasis.

1.4.2.3 FGF23

FGF23 is predominantly expressed in rat fetal osteoblasts, as well as in the thymus, liver and kidney. In mouse models, FGF23 is expressed as early as ED 12.5 in the heart and liver and appears later in the bone.[127] Intact FGF23 levels in the fetus are similar to that of the maternal value during late pregnancy,[102] which is increased from female pre-pregnancy values by two fold.[128]

Although there are no studies of radiolabeled FGF23, it appears that FGF23 does not cross the placenta because *Fgf23* null fetuses have undetectable levels of FGF23, in comparison to their heterozygous mothers which have levels of circulating FGF23 averaging 225 pg/mL.[102] Further to this, WT fetuses borne of mothers that are heterozygous for phosphate regulating endopeptidase homolog x-linked ($Phex^{+/-}$,also known as $Hyp^{+/-}$) mothers, which have an excess of FGF23, have normal levels of circulating FGF23 in comparison to WT fetuses borne of $Ffg23^{+/-}$ mothers, despite the excess in maternal FGF23.[102]

Studies of absence or excess of fetal FGF23 have shown no differences in any measured parameters of bone and mineral homeostasis, including serum and amniotic fluid phosphorus, skeletal mineral content and morphology and placental phosphorus transport.[102] This suggests that FGF23 is not an important regulator of fetal phosphorus homeostasis. Moreover, when WT mothers are challenged with a high phosphorus diet, fetuses maintain normal phosphorus and FGF23 levels, independent of their respective maternal values (unpublished).

There is currently little data on FGF23 in human fetuses. Based on the scant data available, FGF23 in the cord blood appears to be lower than maternal values, however, c-terminal FGF23 values are increased.[128] This may suggest that fetal FGF23 is plentiful in the fetal circulation, but is being rapidly cleaved which is producing elevated levels of non-functional c-terminal fragments in the circulation. Moreover, levels of Klotho, the co-receptor for FGF23 are increased by six-fold in the cord blood in comparison to adult or neonatal values.[129] With little human data available, we must rely on what we know about the mouse model to help us understand FGF23's role in the fetus.

1.4.2.4 Calcitriol

Although 25OHD readily crosses the placenta from mother to fetus, calcitriol typically circulates at less than 50 % of the maternal value in fetal rodents[130-133] and pigs.[80] The lower circulating calcitriol values in fetal mice compared to the maternal circulation may be explained by low levels of plasma PTH, high serum calcium and phosphorus, as well as increased 24OHase activity that are presented under normal fetal physiological conditions.[93]

In spite of playing a critical role in bone and mineral metabolism in both the child and the adult, evidence from both animal models and human disorders indicate that the low levels of circulating fetal calcitriol are not detrimental to fetal mineral homeostasis and skeletal development. Studies of severely vitamin D deficient rats [134-136], *Cyp27b1* null pigs [80] and *Vdr* null mice[131,137] have consistently shown normal fetal serum calcium, phosphorus,

and PTH levels, as well as ash weight and skeletal mineral content. *Cyp27b1* null mice are also normal at birth, but until now, there have been no studies in the literature of their serum chemistries and skeletal mineral content during fetal development.[81,138]

The findings to date suggest that fetal calcium homeostasis and skeletal development and mineralization are independent of calcitriol and its receptor; however, the effect of absence of calcitriol still needs to be studied. The placenta provides calcium to the fetus without relying on calcitriol, and vitamin D deficient and *Vdr* null placentas express normal concentrations of the vitamin D dependent factors calbindin-D-9k and Ca^{2+}-ATPase, which are important for intestinal calcium absorption and calcium homeostasis in the adult. [131,139,140]

The effects of loss of *Vdr* on fetal bone and mineral homeostasis have been studied using two main *Vdr* knockout models; the Boston model and the Leuven model, both named after the cities in which they were developed. In the case of the Boston model, *Vdr* is disrupted by removal of exon 3, encoding the second zinc finger of the *Vdr*-binding domain.[23] Studies of the Boston model, carried out in our lab, have shown that *Vdr* null offspring born of both $Vdr^{+/-}$ and *Vdr* null mothers have normal serum calcium, phosphorus, PTH, ash weight, tibial morphology and mineral content.[131] In the case of the Leuven model, exon 2 of the *Vdr* gene is removed, which encodes the first of two zinc fingers that are characteristic of nuclear receptors and required for DNA binding.[141] The Leuven model results in a truncated VDR which is transcribed, translated and secreted and has abnormal signaling. Thus, the Leuven model is not a complete *Vdr* null. Studies

of this model looked at *Vdr* null fetuses borne of *Vdr* null mothers. Similar to the study of the Boston model, these fetuses also had normal parameters related to bone and mineral homeostasis.[137] Thus, despite not being a true null, the Leuven model ultimately led to a similar phenotype as the Boston model which is a true null.

Human data is consistent with the notion that calcitriol may not be necessary for normal fetal bone and mineral homeostasis. Circulating calcitriol levels in the human fetus mirror those of fetal rodents and pigs, with the fetal calcitriol levels being less than 50 % of the maternal values.[142-146] Calcitriol synthesis in the fetus is thought to be suppressed by the high calcium, phosphorus and low PTH values within the cord blood. Similar to observations from animal models of vitamin D deficiency, babies with severe vitamin D deficiency have normal serum calcium, phosphorus, PTH, and skeletal morphology and mineral content.[93] This evidence from both animal models and human observations indicate that vitamin D, calcitriol or VDR are not required to maintain normal mineral homeostasis and skeletal development within the fetus.

1.5 Renal mineral absorption and the amniotic fluid

In the adult, the kidneys play a vital role in regulation of bone and mineral homeostasis through their ability to adjust the relative reabsorption and excretion of minerals in response to calciotropic and phosphotropic hormones, as well as being the main site of calcitriol synthesis. It is likely that the kidneys play a lesser role in the fetus than the adult given: 1) the placenta plays a dominant role in the handling of mineral and calcitriol

synthesis, and 2) the excreted mineral is not being lost as it is in the adult. Instead, the fetal urine makes up a large part of the amniotic fluid which is swallowed, absorbed and brought back into the fetal circulation. Therefore, calcium and phosphorus filtered by the fetal kidneys can be partially reabsorbed into the circulation to maintain mineral concentrations in the blood.

1.6 Role of the fetal intestines

The contrast in the physiological role of calcitriol after birth compared to during fetal development may be due to the trivial role of the fetal intestines during gestation. Unlike in the adult, where the intestines play a considerable role in mineral metabolism, the fetus receives its mineral through active transport of calcium, phosphorus and magnesium across the placenta from the maternal circulation.[93] The fetal intestines cannot be studied directly. However, pre-term babies (and animals) are the functional equivalent of fetuses, and they show passive absorption of mineral that is not calcitriol-responsive, and low expression of VDR.[93]

1.7 Placental mineral transport

Beyond the intestines, kidneys and skeleton which play prominent roles in the regulation of mineral homeostasis in the adult, the fetus has a unique organ, the placenta, which has a vital role in fetal mineral homeostasis. The placenta assumes many functions that are carried out by other organs in the adult, including acting like the fetal lungs, disposing of fetal waste, hormone production as well as other functions. The placenta is responsible for the transport of calcium and other minerals from the mother to the fetus.[121,147,148]

Placental mineral transport may occur via simple diffusion, paracellular transport, vesicular transport or active transport.[121]

Calcium transport across the placenta is thought to be similar to the passage of calcium across intestinal cells. TRPV6 channels open on the maternal-facing basement membrane of the placenta to allow calcium entry into placental cells, calcium shuttles across to the opposite basement membrane via Calbindin-D_{9k} and is extruded into the fetal circulation by Ca^{2+} ATPase.[93] The roles of these proteins in placental calcium transport are supported by data from fetal animal studies. Fetuses lacking either TRPV6 or PTHrP have reduced placental calcium transport, as well as a decrease in Calbindin-D_{9k}, within the placental yolk sac.[149,150]However, it is not known whether the reduced placental calcium transport is directly or indirectly caused by the decrease in expression of Calbindin-$D_{9k.}$ Ca^{2+} ATPase has also proven to be important in placental calcium transport, as its knockdown leads to reduced calcium transport within the rat placenta.[151,152]

Less is known about phosphorus transport across the placenta. But, it is known that the placenta expresses many phosphorus regulating genes including sodium phosphate transporters *Napi2a, Napi2b, Napi2c*, as well as low expression of *Fgf23.*[102]

1.8 Role of calcitriol in placental function

The role of calcitriol in placental function remains unclear. While the expression of *Vdr*, *Cyp27b1* and calcitriol dependent calcium-transporting factors *Pmca1*, *S100G*, and *Trpv6* in placental trophoblasts suggest calcitriol may play a role in placental function, studies in

vitamin D deficient fetal rats[139] observed normal placental calcium transport. Interestingly, however, when fetal mice are exposed to pharmacological doses of calcitriol, placental calcium transport is increased.[93]

Both the Boston and Leuven *Vdr* knockout models have shown high levels of circulating calcitriol in the *Vdr* null fetuses. [131,137] Interestingly, studies of the Boston model have shown that both *Vdr* null fetuses borne of $Vdr^{+/-}$ and of *Vdr* null mothers display a non-significant trend towards increased placental calcium transport that became significant when results from these studies were pooled.[131] *Vdr* null fetuses from $Vdr^{+/-}$ mothers were also shown to have increased expression of placental *Trpv6* and increased expression of *Pthrp* as demonstrated through both immunohistochemistry and gene expression studies. Similarly, in studies of the Leuven model, *Vdr* null fetuses were shown to have significantly increased placental calcium transport and placental expression of *Trpv6* in comparison to their $Vdr^{+/-}$ littermates, although no differences in placental expression of *Pthrp* were evident.

The higher rate of placental calcium transport in *Vdr* null fetuses of both the Boston and Leuven models may indicate that if calcitriol does have a role, it may be to reduce placental calcium transfer, such that in the absence of VDR, calcium transport increases due to loss of calcitriol's "brake" on the transport of mineral from the maternal to fetal circulation. Alternatively, since *Vdr* null fetuses have higher than normal concentrations of calcitriol,[153,154] and since pharmacological treatment with calcitriol stimulated placental calcium transport in other studies,[93] it is conceivable that calcitriol acts through non-

classical receptors to upregulate placental calcium transport despite the absence of VDR. Therefore, the Vdr models do not definitively address the action of calcitriol.

1.9 Rationale, hypothesis and objectives

It is well known that calcitriol plays a significant role in bone and mineral homeostasis in the adult. However, less is known about the role of calcitriol in fetal bone and mineral homeostasis. Studies from *Vdr* null fetuses suggest that calcitriol is not required, but the high levels of circulating calcitriol and upregulated placental function in these fetuses left uncertainties that may be answered by studying fetuses that cannot synthesize calcitriol.

There are three parts to our hypothesis:

(1) Calcitriol is not required to maintain bone and mineral homeostasis in the fetus.

(2) The upregulation of placental function in the *Vdr* null fetuses may be due to calcitriol having physiological effects to reduce placental calcium transfer, such that when calcitriol has no receptor to signal through, calcium transport increases due to loss of calcitriol's normal effects as a "brake" on the forward flow.

(3) Alternatively, since *Vdr* null fetuses have higher than normal concentrations of calcitriol, it is conceivable that such high levels of calcitriol act through non-classical receptors to upregulate placental calcium transport despite the absence of VDR. Such an action might not be physiological but only brought about with high concentrations of calcitriol, and this would explain why both Vdr null fetal mice and fetal sheep treated with pharmacological doses of calcitriol had increased placental calcium transport.

Hypotheses were tested using a *Cyp27b1* mouse knock out model, which lack the enzyme necessary to synthesize calcitriol. *Cyp27b1* null and WT sisters were mated to *Cyp27b1*$^{+/-}$ males in order to study null and *Cyp27b1*$^{+/-}$ fetuses borne of null mothers, as well as WT and *Cyp27b1*$^{+/-}$ fetuses borne of WT mothers.

The objectives of this research were to determine whether loss of calcitriol in *Cyp27b1* null fetuses of null mothers when compared to their siblings and to fetuses of related WT mothers:

1. Alters serum minerals, calciotropic hormones, or amniotic fluid mineral content.
2. Alters skeletal morphology, ash weight, or skeletal mineral content.
3. Alters the rate of placental ^{45}Ca transport from mother to fetuses.
4. Alters expression of relevant genes within placenta and kidneys.

II. Materials and Methods

2.1 Animal husbandry

2.1.1 Murine model

Cyp27b1 null mice were provided by Dr. René St-Arnaud of McGill University in Montreal, Quebec. The 1αOHase enzyme was inactivated by Cre-mediated excision of the gene in embryonic stem cells. Homologous recombination with the 1αOHase locus and wild-type locus generated a targeted allele in which exon 8, encoding the heme-binding domain, was deleted, effectively generated a null allele.[81] The mice were maintained in the C57BL/6 parent strain, through heterozygous matings, resulting in the generation of offspring of three genotypes: wild type (WT) with both normal *Cyp27b1* alleles (*Cyp27b1*$^{+/+}$), heterozygous (*Cyp27b1*$^{+/-}$) with one normal *Cyp27b1* allele and one *Cyp27b1* allele ablated, and *Cyp27b1* null with both *Cyp27b1* alleles ablated (*Cyp27b1*$^{-/-}$).

In a previous study in our lab, a traditional breeding approach was used in which *Cyp27b1*$^{+/-}$ males and females were mated so that WT, *Cyp27b1*$^{+/-}$ and *Cyp27b1* null fetuses were generated in each litter. However, surprisingly, the *Cyp27b1* null fetuses had detectable levels of circulating calcitriol.

Therefore, in order to obtain truly calcitriol-deleted fetuses, *Cyp27b1* null and WT mothers, first degree relatives of each other, were mated to the same *Cyp27b1*$^{+/-}$ males. This resulted in *Cyp27b1* null and *Cyp27b1*$^{+/-}$ fetuses from *Cyp27b1* null mothers, and *Cyp27b1*$^{+/-}$ and WT fetuses from WT mothers (Figure 9). Comparison of *Cyp27b1* null

and WT fetuses conceivably revealed any effects of calcitriol deficiency, whereas comparison of *Cyp27b1*$^{+/-}$ fetuses from two different maternal backgrounds enabled detection of any maternal influence on the fetal phenotype**.**

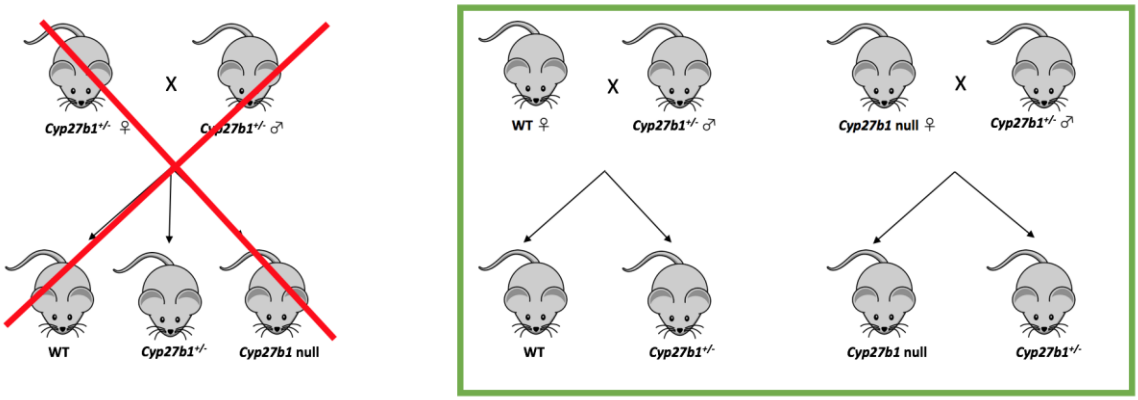

Figure 9: Mating Scheme. The traditional mating scheme of mating Cyp27b1$^{+/-}$ males to *Cyp27b1*$^{+/-}$ females could not be used as null fetuses obtain calcitriol from their HET mother. Therefore, *Cyp27b1*$^{+/-}$ males were mated to either WT or *Cyp27b1* null females in order to produce litters containing WT and *Cyp27b1*$^{+/-}$or *Cyp27b1* null and *Cyp27b1*$^{+/-}$ fetuses respectively.

2.1.2 Rescue diet

Both WT and *Cyp27b1* null female mice were placed on a rescue diet (TekLad TD.94112; Harlan TekLad, Madison, WI) consisting of 2% calcium, 1.25% phosphorus, and 20% lactose.[155] The diet's high lactose content increases paracellular calcium absorption[138] and it has been used in prior studies of *Cyp27b1* null and *Vdr* null mice to normalize fertility.[156] The diet was provided *ad libitum* at weaning to allow for normal bone phenotype and fertility in the null mice.

2.1.3 Timed mating

Virgin first-degree relative pairs of WT and *Cyp27b1* null females were mated with *Cyp27b1*$^{+/-}$ males at approximately 16:00 hours on Thursday evenings. At approximately 9:00 hours the following morning, mice were checked for the presence of a vaginal mucus plug. The presence of a vaginal plug was indicative that the mouse had mated and may have conceived; if so, this was embryonic day (ED) 0.5. However, a plug was not always evident due to the plug being too small or the plug having fallen out. Mice were returned to their original cages and continually mated on Thursday evenings until pregnancy was visually confirmed. The normal gestational period for these mice was 18.5 days.

2.1.4 Animal housing

Animals were housed in a facility operated by Animal Care Services of Memorial University of Newfoundland. Animals were housed in individually ventilated rodent

cages with 501 cm^2 floor area (GM500, Techniplast Canada) and Bed-O-Cobs corn cob absorbent bedding (The Andersons, Maumee, OH, USA). This is in accordance with the Canadian Council on Animal Care (CCAC). The regular light and dark cycle was used with light from 8:00 hours to 20:00 hours.

2.1.5 Animal care approval

All experimental procedures were approved by the Institutional Animal Care Committee (IACC) of Memorial University of Newfoundland.

2.2 Genotyping

2.2.1 Animal identification

At 21 days of age (the time of weaning), experimental mice were weaned from their mothers and separated into cages based on sex, with a maximum of 4 mice per cage. Mice were briefly anesthetized with Isoflurane (Baxter, Deerfield, IL), and their right ear was crimped with a tag for identification purposes.

2.2.2 Tail sample collection

While still under anaesthesia from the ear tagging procedure, a 0.5cm section of tail was clipped from each mouse with a sterile razor blade and placed into a labelled 1.5 mL Eppendorf tube. To digest tail clippings, 300 µl of cell lysis solution and 1.5 µl of proteinase K (Invitrogen, Carlsbad, CA) was added to each tube and tubes were placed in

an incubator (Thermo Fisher Scientific, Burlington, ON) for 18-24 h at 55 ˚C. Following this incubation period, tails were fully digested and ready for DNA extraction.

2.2.3 DNA extraction

DNA extraction was completed as per the Qiagen Purgene® Core A Kit (Qiagen, Toronto, ON). A protein precipitation solution (100 ul) was added to each digested tail sample and each sample was then vortexed for 20 sec at high speed. Samples were then centrifuged at 16,000 x *g* for 3 min in an IEC Micromax centrifuge (Thermo Fisher Scientific, Burlington, ON). Following centrifugation, each tube was checked for the presence of a white protein pellet and 300 µl of supernatant from each sample was pipetted into fresh 1.5 mL microcentrifuge tubes containing 300 µl of isopropanol. Tubes were gently inverted 50 times to precipitate the DNA, and then centrifuged for 1 min at 16,000 x *g* to obtain the DNA in a small white pellet at the bottom of each tube. Following centrifugation, the supernatant was decanted and 300 µl of 70 % ethanol was added to each sample. Tubes were then inverted several times in order to wash the DNA pellet, following which they were centrifuges for 1 min at 16,000 x *g*. The supernatant was again decanted and excess ethanol was removed with a pipette. Tubes were left to air dry for 5 minutes to further allow the ethanol to evaporate off. Finally, 200µl of DNA hydration solution was added to each sample, samples were vortexed for 5 seconds and incubated at 65 degrees Celsius (ºC) for 1 hour in order to dissolve the DNA.

2.2.4 Polymerase chain reaction (PCR)

PCR was carried out on the extracted DNA. In order to distinguish *Cyp27b1* null, *Cyp27b1*$^{+/-}$ and WT mice, a 3-primer system was used:

Cyp27-1 (forward): 5' - AAT TCC CGT CCA GAC AGA GAC ATC C – 3'

Cyp27-2 (reverse): 5' – GGT CAT GGG CTT GAT AGG AGC ACC – 3'

Cyp27-3 (reverse): 5' – GGG TGG GGA ATG TGA AGA AGA GGA TCT G – 3'

The PCR master mix was made with 10x PCR reaction buffer, deoxyribose nucleotide tri-phosphates (dNTPS - dATP, dTTP, dCTP, dGTP), primers (*Cyp27-1*, *Cyp27-2*, *Cyp27-3*), 50 mM $MgCl_2$, Platinum Taq DNA polymerase, and distilled water (Invitrogen, Carlsbad, CA). The PCR master mix was aliquoted into fresh 0.2 mL PCR tubes (Thermo Fisher Scientific, Burlington, ON) with 19.5 µl in each. Next, 2 µl of DNA sample was then added into the solution in each tube. PCR tubes were then placed into Bio-Rad C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, California).

The PCR running program consisted of 7 steps, described as follows: Step 1: 94 °C for 5 min for initial denaturation, Step 2: 94 °C for 30 sec to denature the DNA, Step 3: 58 °C for 30 sec for annealing, Step 4: 72 °C for 30 sec for elongation of complementary strand, Step 5: return to step 2 and repeat for 35 cycles for continued amplification, Step 6: 72 °C for 10 min to elongate any remaining strands and Step 7: 4 °C infinitely for storage of reaction.

2.2.5 Gel electrophoresis

Gel electrophoresis was completed in order to separate and display PCR products. A 1.2 % agarose gel was made using 10 mL TAE buffer (0.12 M EDTA, 0.40 M Tris, 11.5 % Glacial Acetic Acid, pH 8), 90 mL deionized water, 1.2 g agarose (Invitrogen, Carlsbad, CA) and 10 µl of SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA). Agarose powder was added into the TAE buffer solution and then melted by microwaving in two separate 1 min intervals. Next, SYPBR safe gel stain was added to the solution and gently swirled. The solution was poured into the gel casting tray, containing 2 gel comb inserts, and allowed to solidify. While the gel was solidifying, 4 µl of gel loading dye [1mL Tris, 0.03 g bromophenol blue, 0.03 g xylene cyanol FF, 60 mL glycerol, 12 mL of 0.5 M EDTA, 27 mL deionized water (dH20)] was added to each PCR tube.

Once the gel had solidified, gel running buffer [90 mL 10x TAE and 810 mL dH20] was added to the gel electrophoresis chamber and gel comb inserts were carefully removed. Fifteen µl of PCR product and gel dye mixture was injected into each well of the gel. The samples were run at 200 V for 20 min.

Following electrophoresis, the bands were visualized under UV light using the Kodak Gel Doc System (Bio-Rad, Hercules, CA). The results were analyzed using Bio-Rad Image lab software (Bio-Rad, Hercules, CA). A single 250 bp band indicated WT mice, a single 350 bp band indicated null mice and bands at both 250 bp and 350 bp indicated HET mice (Figure 10).

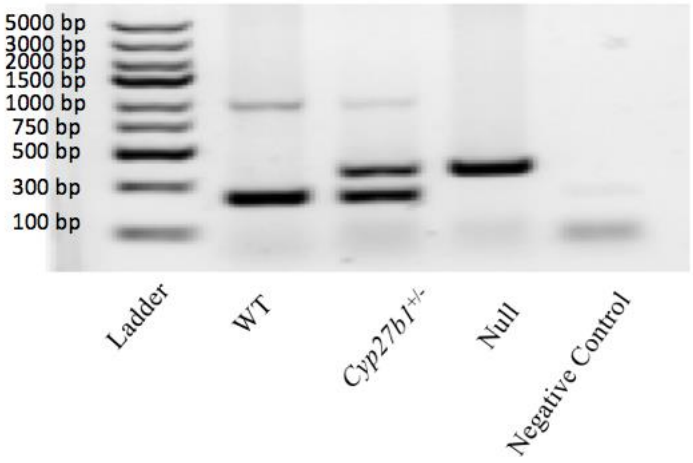

Figure 10: Genotyping by Polymerase Chain Reaction and Gel Electrophoresis. Sample PCR products run on a 1.2 % agarose gel. The presence of a single band at 250 bp indicates a WT mouse, presence of both 250 bp and 350 bp bands indicates a *Cyp27b1*$^{+/-}$ mouse, and the presence of a single 350 bp band indicates a *Cyp27b1* null mouse.

2.3 Sample collection and storage

2.3.1 Serum collection

On ED 18.5, maternal blood serum samples were collected by bleeding the tail vein. A small clipping of tail was cut with a sterile razor blade and the blood was massaged out into a 0.6 mL micro centrifuge tube (Thermo Fisher Scientific, Burlington, ON). Under brief anesthesia using Isoflurane (Baxter, Deerfield, IL), mothers were then euthanized via cervical dislocation. Fetuses were removed via Caesarean section (C-section) and were detached from their placentas by severing their umbilical cords. Fetal blood was collected by making a small incision in the carotid artery and jugular vein using a sterile razor blade and blood was collected into a Micro-Hematocrit Capillary Tube (Fisher Scientific, Burlington, ON). Following fetal blood collection, fetuses were immediately euthanized by decapitation. Blood samples were subsequently spun on a microcentrifuge for 5 min at 16,000 x *g* to separate out serum from clotted blood. Serum was stored at -20 °C for future analysis.

2.3.2 Amniotic fluid collection

On ED 17.5, mothers were sacrificed by cervical dislocation and pups were removed by C-section. Each amniotic sac was lanced using a 20G needle (BD PrecisionGlide™) and the fluid was collected in 60 µl micro-hematocrit capillary tubes (Thermo Fisher Scientific, Burlington, ON). Samples were then transferred to 0.6 ml microcentrifuge tubes (Fisher Scientific, Burlington, ON) and stored at – 20 °C until analysis.

2.3.3 Collection of placenta and fetal kidneys

On ED 18.5, following detachment of the fetus from the placenta, placentas were individually placed into 1.5 mL Eppendorf tubes and were flash frozen in liquid nitrogen. Serum was then collected and fetuses were euthanized. Following euthanasia, fetuses were laid in a supine position and a small incision was made from the pelvic bone to the sternum. Both kidneys were then removed from each fetus, placed into a 1.5mL Eppendorf tube and flash frozen in liquid nitrogen. Tissues were stored at -70 ºC for future analysis.

2.3.4 Collection of whole bodies

On ED. 18.5, following removal of fetal kidneys, fetal bodies were placed in labelled scintillation vials containing 10 % buffered formalin (1x PBS, 4 % Formaldehyde, dH2O) and stored at room temperature. Once all samples had been collected, fetuses were removed from vials, the right hind limb was removed and placed back into the buffered formalin for embedding in paraffin.

2.4 Analysis of serum and amniotic fluid mineral content

2.4.1 Serum and amniotic fluid total calcium measurement

Serum (ED 18.5) and amniotic fluid (ED 17.5) total calcium were measured using a calcium assay (Sekisui Diagnostics, Charlottetown, PEI). The assay is based on the principle that Arsenazo III reacts with calcium to form a complex that is blue-purple in color with a maximum absorbance of 650 nm. Therefore, results read at this wavelength are directly proportional to the total calcium concentration in the sample. The reportable

range for this kit was 0.01 mmol/L to 3.75mmol/L. Serum and amniotic fluid samples were measured undiluted and following kit protocol. A spectrophotometer (Ultraspec 2000; Pharmacia Biotech, Piscataway, NJ) was used to measure samples at an absorbance of 650 nm. Deionized water was used as a blank.

2.4.2 Serum and amniotic fluid inorganic phosphate measurement

Serum (ED 18.5) and amniotic fluid (ED 17.5) inorganic phosphate were measured using a Phosphorus-SL assay (Sekisui Diagnostics, Charlottetown, PEI). The assay is based on the principle that inorganic phosphorus reacts with ammonium molybdate in the presence of sulfuric acid to produce an unreduced phosphomolybdate complex. The concentration of inorganic phosphorus in the sample is directly proportional to the absorbance at 320 nm. The reportable range of the kit was 0.03 mmol/L to 6.46 mmol/L. Serum and amniotic fluid samples were measured undiluted and following kit protocol. A spectrophotometer (Ultraspec 2000; Pharmacia Biotech, Piscataway, NJ) was used to measure samples at an absorbance of 320 nm. Deionized water was used as a blank.

2.5 Hormone physiology

2.5.1 Serum calcitriol

Serum calcitriol was measured using a 1,25-dihydroxy vitamin D enzyme-immunoassay (EIA) Kit (Immunodiagnostic Systems, Maryland, USA). The detection limit for the assay in human serum was 6 pmol/L. The manufacturer has not published the detection limit in mice. The assay was carried out over a two-day period. The calcitriol in the samples was first purified by immunoextraction then quantified by EIA. The principle of

the assay was based on competitive binding of either free calcitriol or calcitriol linked to biotin for a limited amount of highly specific sheep anti-calcitriol antibody binding sites. The amount of complexed biotin bound to the anti-sheep antibody was inversely proportional to the concentration of free calcitriol. Due to the amount of fetal serum needed for each sample, the assay was carried out using a 1:2 dilution of serum in PBS supplemented with 1% BSA. Maternal and fetal serum samples were measured. The remaining assay procedure followed kit protocol for "alternative sample preparation" and remaining immunoextraction assay procedure. Any values that were below the assay sensitivity were reset to values that equaled the assay's detection limit.

2.5.2 25-Hydroxyvitamin D and 24,25-dihydroxyvitamin D

Maternal and fetal serum were sent to Dr. Glenville Jones at Queen's University in Kingston Ontario for the measurement of 25-OHD_3 and 24,25-dihydroxyvitamin D_3. Samples were measured using liquid chromatography tandem mass spectrometry. The assay methods, details and procedure have been previously published. [157]

2.5.2 Serum parathyroid hormone (PTH)

Serum PTH was measured using a Mouse Intact PTH ELISA Kit (Immunotopics Inc., San Clement, CA). The detection limit of the assay was 4 pg/mL. The assay principle is a two-site enzyme-linked immunosorbent assay (ELISA) . Samples were incubated simultaneously with a biotinylated capture antibody and a horseradish peroxidase (HRP)

conjugated antibody in a streptavidin coated microtiter well. Intact PTH contained in the sample is immunologically bound by the capture antibody and the detection antibody to form a sandwich complex. The enzymatic activity of the antibody complex bound to the well is directly proportional to the amount of PTH in the sample. Samples were measured using an Epoch Microplate Spectrophotometer (Biotek) at an absorbance of 450 nm. Maternal and fetal serum collected on ED 18.5 were measured and experiments were completed as per kit protocol. Any values that appeared to be below the assay sensitivity were reset to values that equaled the assay's detection limit.

2.5.3 Serum fibroblast growth factor 23 (FGF23)

Serum FGF23 was measured using a FGF23 ELISA Kit (Kainos Laboratories, INC., Tokyo, Japan) designed for measurement of mouse serum. The detection limit of the assay is 3 pg/mL. The assay principle is a two-step enzyme-linked immunosorbent assay. In the first reaction, samples are incubated with the immobilized antibody in a microtiter well, allowing the FGF23 in the samples to be captured by the antibody. In the second reaction, the immobilized FGF23 is incubated with HRP labelled antibody to form a sandwich complex. The enzymatic activity of the antibody complex bound to the well is directly proportional to the amount of FGF23 in the sample. Samples were measured using an Epoch Microplate Spectrophotometer (Biotek, Winooski, VT). Maternal and fetal serum collected on ED 18.5 were measured and experiments were completed as per kit protocol. Any values that appeared to be below the assay sensitivity were reset to values that equaled the assay's detection limit.

2.6 Histomorphometry

Undecalcified, paraffin embedded hind limbs, collected from WT and Cyp27b1 null fetuses, were sectioned into 5 μm sections with a Leica RM2135 rotary microtome (Leica Microsystems, Wetzlar, Germany). Tibia sections were placed on a slide warmer (Fisher) for 30 min and samples were then deparafinized and rehydrated using the following protocol: Step 1: Xylene for 2 min (2x), Step 2: 100 % Ethanol (EtOH) for 2 min (2x), Step 3: 95 % EtOH for 2 min, Step 5: 70 % EtOH for 2 min, Step 6: 50 % EtOH for 2 min and Step 7: distilled water for 1 min. Sections were then stained with methyl green (Sigma, Kawasaki, Japan) for 15 min. Excess dye was blotted from the slides, washed in 1-butanol (Fisher Scientific, Burlington, ON) for 10 sec (2x), and then washed in xylene for 10 sec (2x). Cover slips were then mounted on each slide using Permount (Fisher Scientific, Burlington, ON). Stained tibial sections were viewed using a Leica DM500 LED Optical Microscope at 10x magnification (Leica, Wetzler, Germany). Images were taken using a Sony DXC-S500 color digital camera (Sony, Tokyo, Japan).

2.7 Fetal ash and mineral content

Individual fetuses were weighed, placed in crucibles and reduced to ash in a furnace at 500 °C for 24 h. Following cremation, the ash was removed from the crucibles using a fine paintbrush and weighed. The ash weight reflects the amount of total mineral present in the fetal skeleton. Ash samples were then transferred into acid washed 20mL glass scintillation vials and stored at room temperature until samples were prepared for analysis. Before analysis, 253 µl of nitric acid was added to each scintillation vial

containing fetal ash and samples were left at room temperature for 5 days to dissolve. Following dissolution of the ash, 9.75 mL of deionized water was added to each vial. Skeletal calcium and magnesium content were measured using the 2380 Atomic Absorption Spectrophotometer (Perkin –Elmer, Waltham, MA). Appropriate dilutions were used to allow the reading to be within the proper calibration range. Skeletal phosphorus content was measured using a Phosphorus-SL assay (Sekisui Diagnostics, Charlottetown, PEI) as per kit protocol.

2.8 Gene expression

2.8.1 RNA extraction

RNA was extracted from placentas and fetal kidneys using the RNeasy® Lipid Tissue Kit (QIAGEN, Toronto, ON). Tissues were homogenized in bead tubes containing 10 beads and QIAzol Lysis Reagent (QIAGEN, Toronto, ON) using Precellys® Tissue Homogenizer. Following homogenization, chloroform was added to each sample, and samples were centrifuged to separate homogenate into aqueous and organic phases. The upper, aqueous phase was then collected, and 70 % ethanol was added to provide appropriate binding conditions. The sample was then applied to an RNeasy spin column, where total RNA binds to the membrane, and phenol and other contaminants are removed through washing with buffer RW1, and with buffer RPE (2x). RNA was then eluted in RNase-free water. The RNeasy® Lipid Midi Tissue Kit (QIAGEN, Toronto, ON) was used for extraction of RNA from placentas (≤250 mg of tissue), while the RNeasy® Lipid Mini Tissue Kit was used for extraction of kidney RNA (≤ 30 mg of tissue).

2.8.2 Synthesis of complementary DNA (cDNA)

cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Burlington, ON) using 2 µg of mouse placental or fetal kidney mRNA. Experimental procedure followed kit protocol.

The synthesis program conditions were as follows: Step 1: 25 °C for 10 min; Step 2: 37 °C for 120 min; Step 3: 85 °C for 5 min; Step 4: hold at 4 °C for infinity. Synthesis program was carried out using a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad laboratories, Hercules, California).

2.8.3 Real –time quantitative reverse transcriptase-PCR (RT qPCR)

RT qPCR was completed using the following Taqman® Gene Expression Assays (ThermoFisher Scientific, Burlington, ON): *Cyp27b1*, *Cyp24a1*, *Pmca1*, *VDR*, *S100G*, *Pthrp*, *Trpv6*, and *NaPi 2a*, *NaPi 2b* and *NaPi 2c*. These assays are pre-designed and pre-optimized with a fluorogenic probe with a FAM™ dye label on the 5' end and a non-fluorescent quencher on the 3' end. When targeted cDNA was amplified, the MGB probe was digested by DNA polymerase and the FAM reporter dye was released. Fluorescence from free FAM reporter dye was detected by the real-time PCR system. The intensity of fluorescence was directed to the concentration of targeted cDNA within proper range. The thermal cycler protocol consisted of the following steps: Step 1: 50 °C for 2 min for Uracil-N-Glycosylase (UNG) incubation; Step 2: 95 °C for 20 sec for polymerase activation; Step 3: 95 °C for 1 sec to denature; Step 4: 60 °C for 20 sec for annealing; Step 5: Return to step 3 and repeat 40 times. Multiplex qPCR reactions (gene of interest multiplexed with reference gene) were run in triplicate on the ViiA™ 7 Real-Time PCR

System (Fisher Scientific, Carlsbad, CA). Technical replicates (triplicate) of each sample were used with a reaction volume of 20 µl. Relative expression was determined from the threshold cycle (C_T) normalized to the reference gene (*Gapdh). Gapdh* was labelled with Vic reporter dye, and was measured using a Taqman® Gene Expression Assay.

2.9 Placental calcium transport

On ED 17.5, mothers were briefly anesthetized with Isoflurane and received an intracardiac injection of 100 µl of ^{45}Ca and ^{51}Cr-EDTA cocktail. EDTA is passively transferred and serves as a blood diffusional marker. The cocktail was made up of 1.85 megabecquerels (MBq) of ^{45}Ca, 1.85 MBq of ^{51}Cr and the remaining volume of saline. After 5 min, mothers were sacrificed by cervical dislocation and fetuses were removed via C-section. Tails from each fetus were collected for genotyping. Fetuses were sacrificed by pithing the skulls and were placed in plastic tubes for measurement of radioactivity. The ^{51}Cr activity was measured using a 1480 WIZARD 3 automatic gamma counter (Perkin Elmer, Waltham, MA). Fetuses were then transferred into scintillation vials containing 10 mL of Scintigest and placed in an incubator at 55 ˚C for 24-48 h to solubilize. Following the incubation period, 10 mL of scintillation fluid and 5 drops of glacial acetic acid were added to each vial and vials were covered in aluminum foil and placed in the dark for 24 h to prevent bioluminescence.[125] The activity of ^{45}Ca was measured with a LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Brea, CA). Placental calcium transport was expressed as a ratio of ^{45}Ca/^{51}Cr in each fetus, normalized to the mean value of heterozygous fetuses within each litter.

2.10 Statistical analysis

Data were analyzed using StatPlus: Mac Professional 2009, Build 6.0.3 (AnalystSoft Inc., Vancouver, BC). ANOVA (analysis of variance) was used for analysis of biochemical, transport, and ash data, with Tukey-Kramer post-hoc to determine which pairs of means differed significantly. qPCR data was analyzed using the Comparative C_T method $(2^{\Delta CT})$[158]. All data are presented with mean ± standard error (SE) for fetal data except mean ± standard deviation (SD) for qPCR. On the graphs, significant differences are marked, and the number of observations is indicated in parentheses on the x-axis.

III. RESULTS

3.1 Litter size

The number of fetuses present in WT and *Cyp27b1* null mothers was determined at ED 18.5. It is an indicator of fertility and the capacity of the mother to carry fetuses to term. Litter sizes were no different between the maternal genotypes, specifically 8.3±0.5 in *Cyp27b1* null vs. 8.1±0.3 in WT mothers.

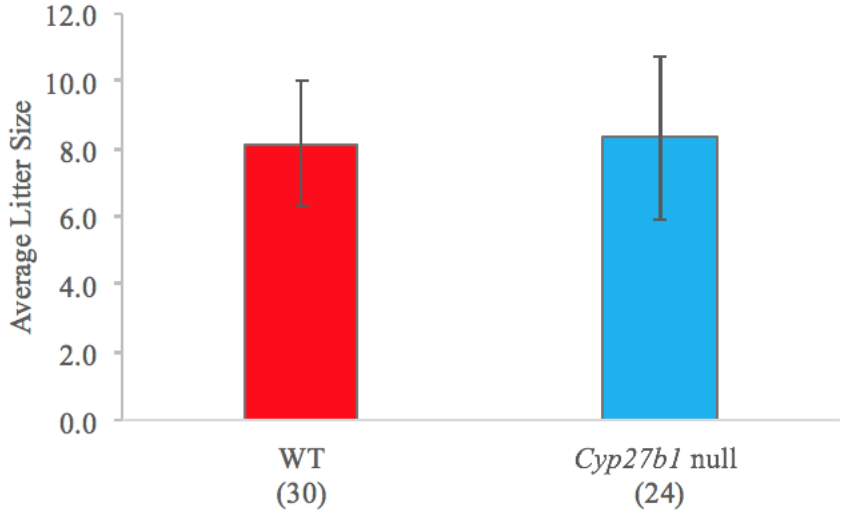

Figure 11: Average litter size of fetuses borne of WT and *Cyp27b1* null mothers. Litter sizes were no different between litters borne of *Cyp27b1* null mothers and litters borne of WT mothers. Values are means ± SE and the number of observations are indicated in parentheses.

3.2 Hormone physiology

The fetal serum concentrations of several hormones relevant to calcium and phosphorus homeostasis were determined.

3.2.1 Calcitriol

We measured calcitriol first, in order to confirm that it is absent in *Cyp27b1* null fetuses born of *Cyp27b1* null females.

Serum calcitriol levels in null fetuses were 16 pmol/L was not significantly different from the published detection limit for human sera (6 pmol/L). It may represent the detection limit for mouse sera, which has not been formally established (Figure 12). The kit uses anti-mouse antibodies, and as a consequence, non-specific binding within mouse sera can be expected to raise the detection limit.

Cyp27b1 null mothers had detectable calcitriol (167 ± 70 pmol/L), but significantly lower than levels found in related WT mothers (719 ± 79 pmol/L).

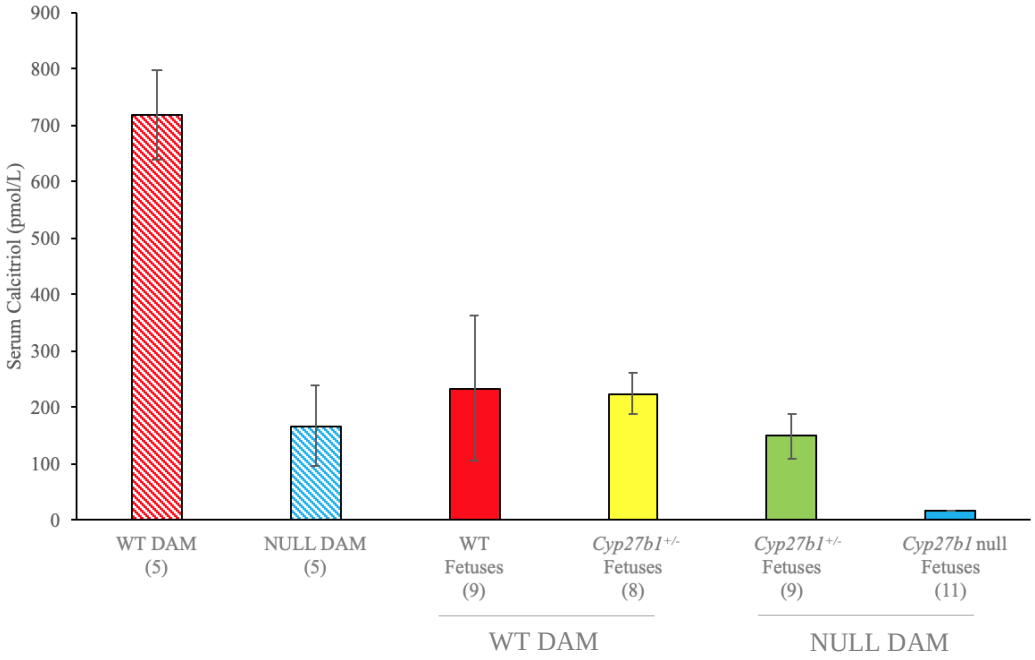

Figure 12: Serum calcitriol in WT and *Cyp27b1* null mothers and their fetuses. *Cyp27b1* null fetuses have levels of calcitriol that are lower than the expected detection limit. Maternal calcitriol is lower in null mothers compared to WT mothers. The dashed line represents the expected detection limit of the assay for mouse sera. Values are means ± SE and the numbers of observations are indicated in parentheses.

3.2.2 25OHD and 24,25-dihydroxyvitamin D

Calcitriol is formed by hydroxylation of 25OHD, and both 25OHD and calcitriol are catabolized by 24-hydroxylation. In this process 25OHD is converted to the inactive form, 24,25-dihydroxyvitamin D. Calcitriol stimulates its own catabolism via Cyp24a1. The absence of calcitriol in *Cyp27b1* null fetuses could result in accumulation of its precursor or altered rate of catabolism by Cyp24a1. Therefore, we measured these metabolites of vitamin D as well, specifically the D_3 isoforms.

Both $25OHD_3$ and 24,25-dihydroxyvitmain D were no different between *Cyp27b1* null fetuses and their *Cyp27b1*$^{+/-}$ littermates. However, these metabolites were significantly higher in these fetuses in comparison to both WT and *Cyp27b1*$^{+/-}$ fetuses borne of WT mothers.

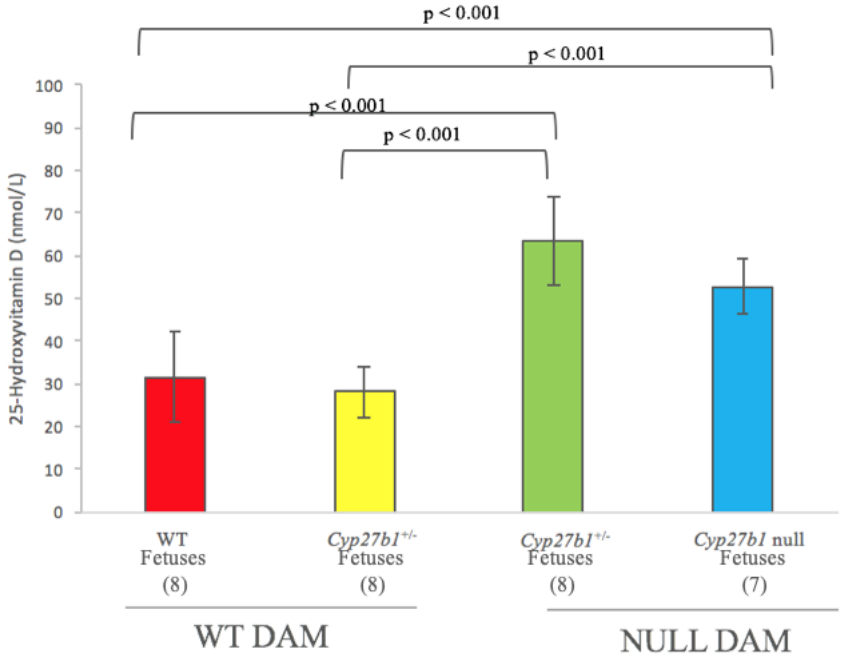

Figure 13: Serum 25OHD$_3$. Serum 25OHD$_3$ was significantly higher in fetuses born of null mothers when compared to fetuses born of WT mothers, whereas there were no differences between litters. This suggests that maternal calcitriol may be an important direct or indirect determinant of fetal 25OHD$_3$. Values are means ± SE and the numbers of observations are indicated in parentheses.

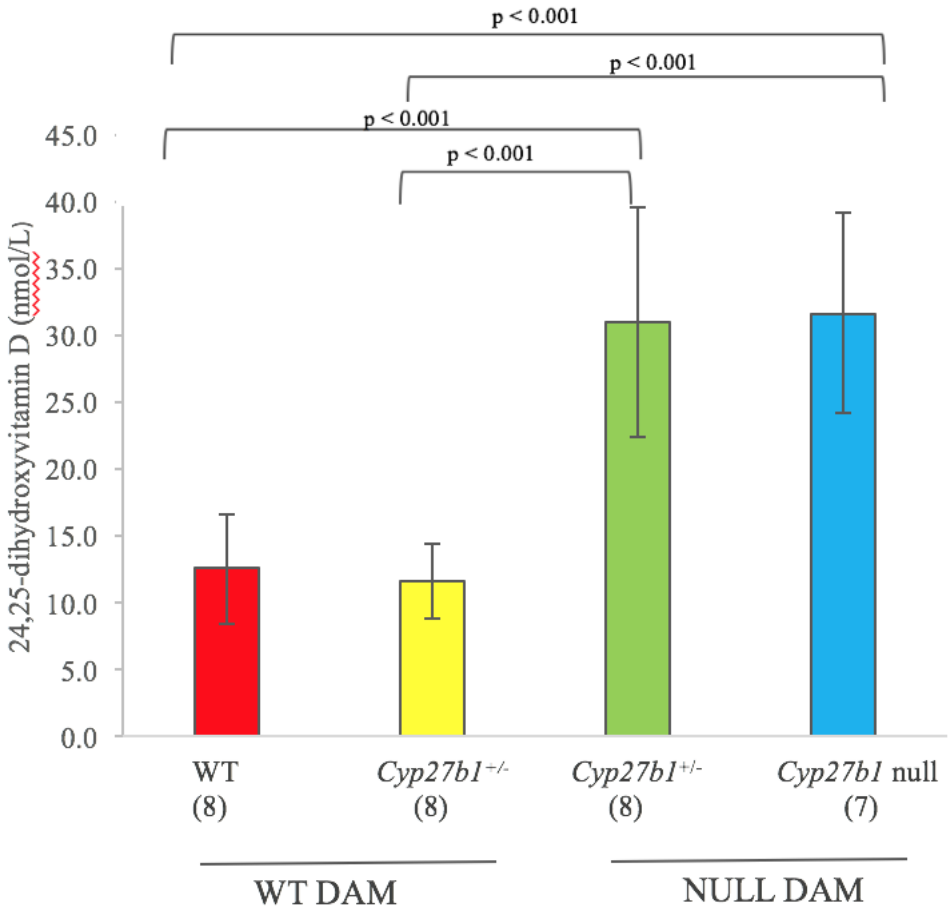

Figure 14: Serum 24,25-dihydroxyvitamin D. Serum 24,25-dihydroxyvitamin D was significantly higher in fetuses born of null mothers when compared to fetuses born of WT mothers, whereas there were no differences between litters. This suggests that maternal calcitriol may be an important direct or indirect determinant of fetal 24,25-dihydroxyvitamin D. Values are means ± SE and the numbers of observations are indicated in parentheses.

3.2.3 PTH

Next, we wanted to measure PTH, as it is the main stimulator of calcitriol's synthesis.

There were no differences in PTH within or between litters. However, fetal serum PTH levels did display a nonsignificant trend towards lower values in fetuses borne of *Cyp27b1* null mothers, in comparison to fetuses borne of WT mothers. This suggests that maternal calcitriol plays a direct or indirect role in regulating fetal PTH levels. I am drawing your attention to this trend now, to support other results that follow.

This data matches prior study of *Vdr* null fetuses which also had normal PTH levels in comparison to their WT counterparts. [131,137]

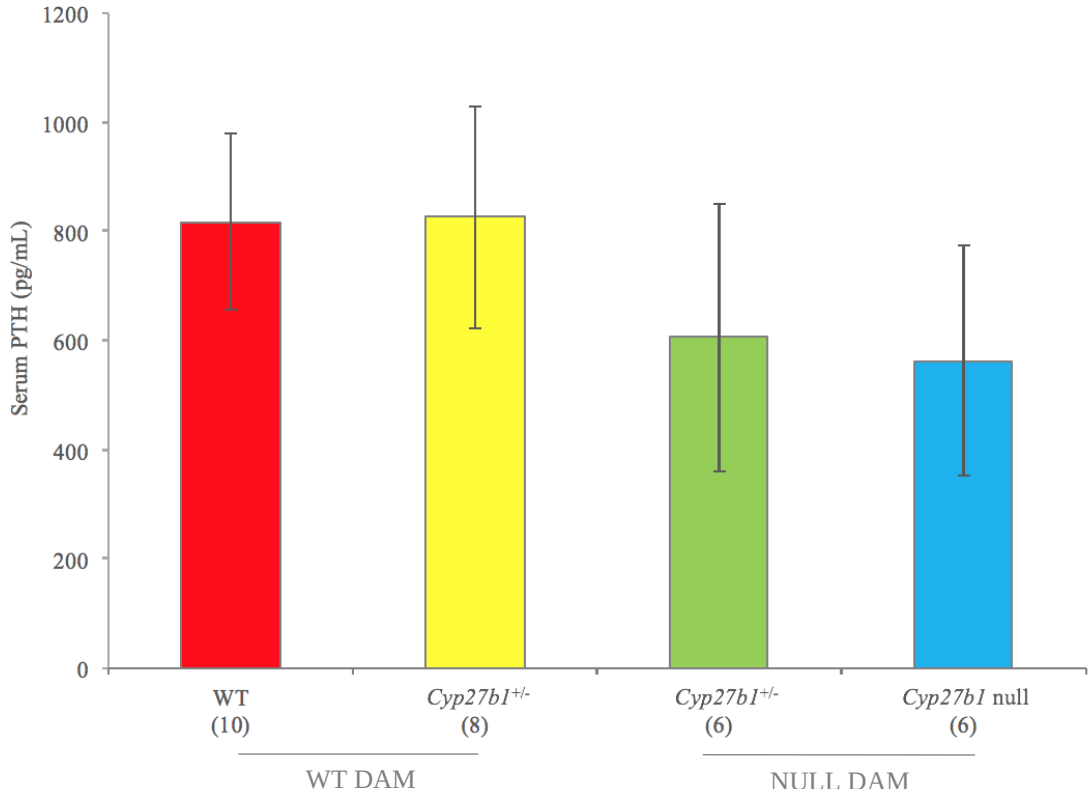

Figure 15: Serum PTH in fetal mice borne of WT and *Cyp27b1* null mothers. Serum PTH is no different between null and *Cyp27b1*$^{+/-}$ fetuses of null mothers, or when compared to WT and *Cyp27b1*$^{+/-}$ fetuses of WT mothers. Values are means ± SE and the numbers of observations are indicated in parentheses.

3.2.4 FGF23

Calcitriol is a potent stimulator of FGF23 synthesis, and so loss of calcitriol's actions should also lower serum FGF23 levels. FGF23 was not significantly different between *Cyp27b1* null fetuses and their *Cyp27b1*$^{+/-}$ littermates. However, both null fetuses and their *Cyp27b1*$^{+/-}$ littermates had significantly lower serum FGF23 than in *Cyp27b1*$^{+/-}$ fetuses born to WT mothers (Figure 16). This suggests that fetal calcitriol is not necessary for regulation of fetal FGF23. Instead, maternal calcitriol may directly or indirectly play a role in determining the fetal FGF23 concentration.

This data contrasts with prior study of *Vdr* null fetal mice which indicated 50% lower serum FGF23 than their WT sisters.[102]

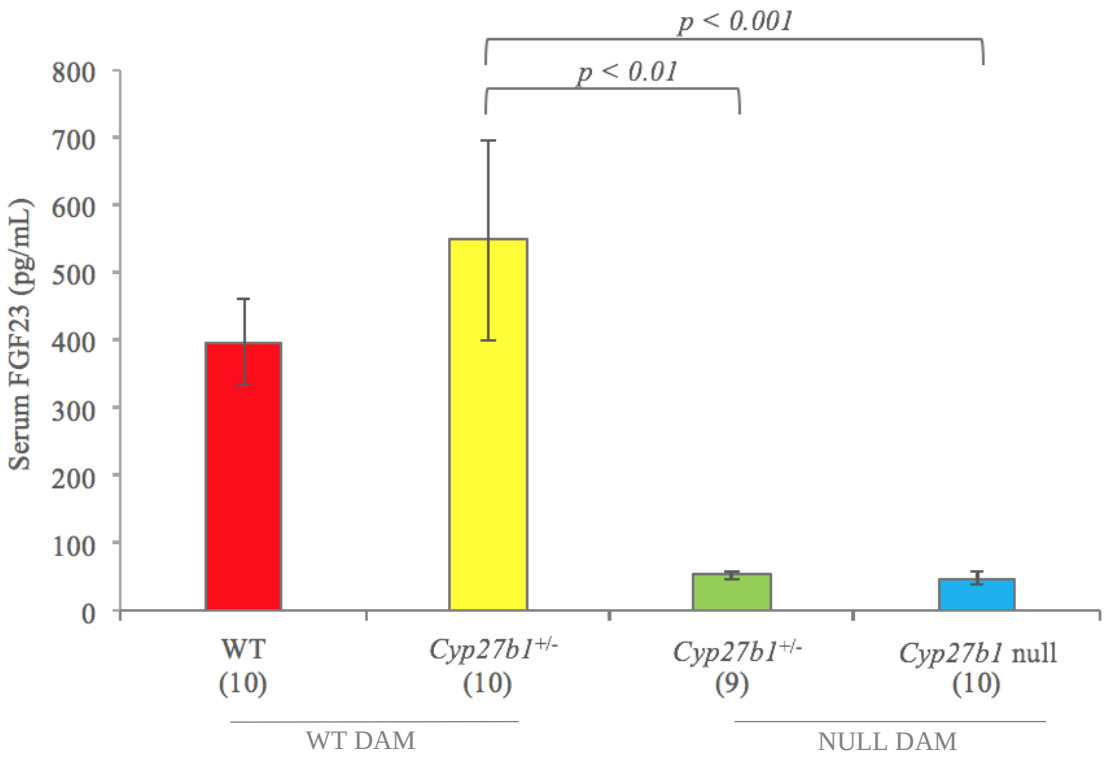

Figure 16: Serum FGF23 in fetal mice borne of WT and *Cyp27b1* null mothers. FGF23 is lower in fetuses born of null mothers compared to *Cyp27b1*$^{+/-}$ fetuses born of WT mothers, whereas there were no differences within litters. This suggests that maternal calcitriol may be an important direct or indirect determinant of fetal FGF23. Values are means ± SE and the numbers of observations are indicated in parentheses.

3.3 Serum and amniotic fluid mineral concentrations

Postnatally, in the absence of calcitriol, serum calcium and phosphorus become low, urine calcium becomes low and phosphorus excretion is increased. We wanted to investigate this in the fetus by determining amniotic fluid calcium and phosphorus values, which acts as a surrogate of renal excretion into the urine.

3.3.1 Total serum calcium and amniotic fluid calcium measurement

Serum calcium was no different between null fetuses and their *Cyp27b1*$^{+/-}$ littermates or when compared to *Cyp27b1*$^{+/-}$ and WT fetuses of WT mothers (Figure 17).

Amniotic fluid calcium was no different between *Cyp27b1* null fetuses and their *Cyp27b1*$^{+/-}$ littermates; however, the value was significantly increased in fetuses from *Cyp27b1* null mothers as compared to the offspring of WT mothers (Figure 18). The normal amniotic fluid in the *Cyp27b1* null fetuses in comparison to their *Cyp27b1*$^{+/-}$ littermates likely indicates that renal calcium excretion is normal. However, as both *Cyp27b1* null and *Cyp27b1*$^{+/-}$ fetuses have increased amniotic fluid calcium in comparison to fetuses borne of WT mothers, maternal calcitriol may be an indirect or director determinant of amniotic fluid calcium. This also suggests that the trend towards lower PTH in fetuses from *Cyp27b1* null mothers may be true, as lower PTH would be expected to lead to an increase in calcium excretion into urine and amniotic fluid, in keeping with what happens postnatally in the presence of low PTH.

This data coincides with prior study of Vdr null fetuses, that found normal amniotic fluid calcium in Vdr null fetuses in comparison to littermates. [131]

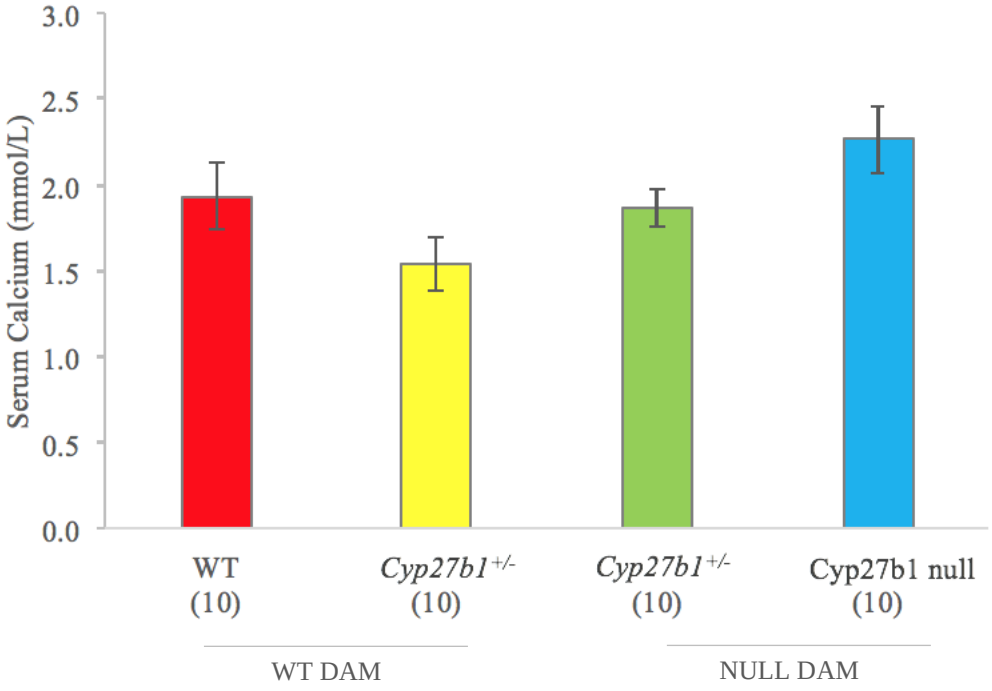

Figure 17: Serum calcium in fetal mice borne of WT and *Cyp27b1* null mothers. Serum calcium is no different between null and *Cyp27b1*$^{+/-}$ fetuses born of *Cyp27b1* null mothers and WT and *Cyp27b1*$^{+/-}$ fetuses born of WT mothers. Values are means ± SE and the numbers of observations are indicated in parentheses.

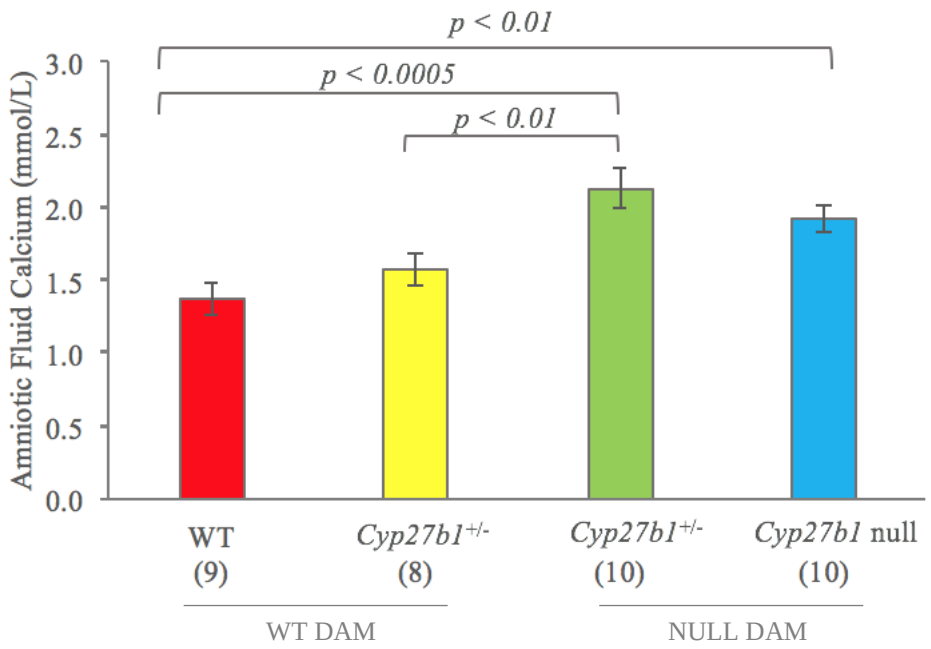

Figure 18: Amniotic fluid calcium in fetal mice borne of WT and *Cyp27b1* null mothers. Amniotic fluid calcium is significantly higher in fetuses born of *Cyp27b1* null mothers compared to fetuses born of WT mothers. This suggests that maternal calcitriol is an important indicator of amniotic fluid calcium content. Values are means ± SE and the numbers of observations are indicated in parentheses.

3.3.2 Serum and amniotic fluid phosphorus measurement

There was no difference in serum phosphorus concentrations between *Cyp27b1* null and *Cyp27b1*$^{+/-}$ littermates or when compared to fetuses borne of WT mothers (Figure 18).

Amniotic fluid phosphorus was no different between null and *Cyp27b1*$^{+/-}$ littermates. However, amniotic fluid phosphorus was significantly lower in fetuses from *Cyp27b1* null mothers, as compared to fetuses from WT mothers (Figure 19). The normal amniotic fluid phosphorus in the *Cyp27b1* null fetuses in comparison to their *Cyp27b1*$^{+/-}$ littermates likely indicates that renal phosphorus excretion is normal. However, as both *Cyp27b1* null and *Cyp27b1*$^{+/-}$ fetuses have decreased amniotic fluid phosphorus in comparison to fetuses borne of WT mothers, maternal calcitriol may be an indirect or director determinant of amniotic fluid phosphorus. Moreover, these results also match the trend towards lower PTH in *Cyp27b1* null fetuses, as lower PTH would cause a decrease in phosphorus excretion into urine and, thereby, amniotic fluid.

This data is consistent with prior study of *Vdr* null fetuses had normal amniotic fluid phosphorus in comparison to their littermates.[131]

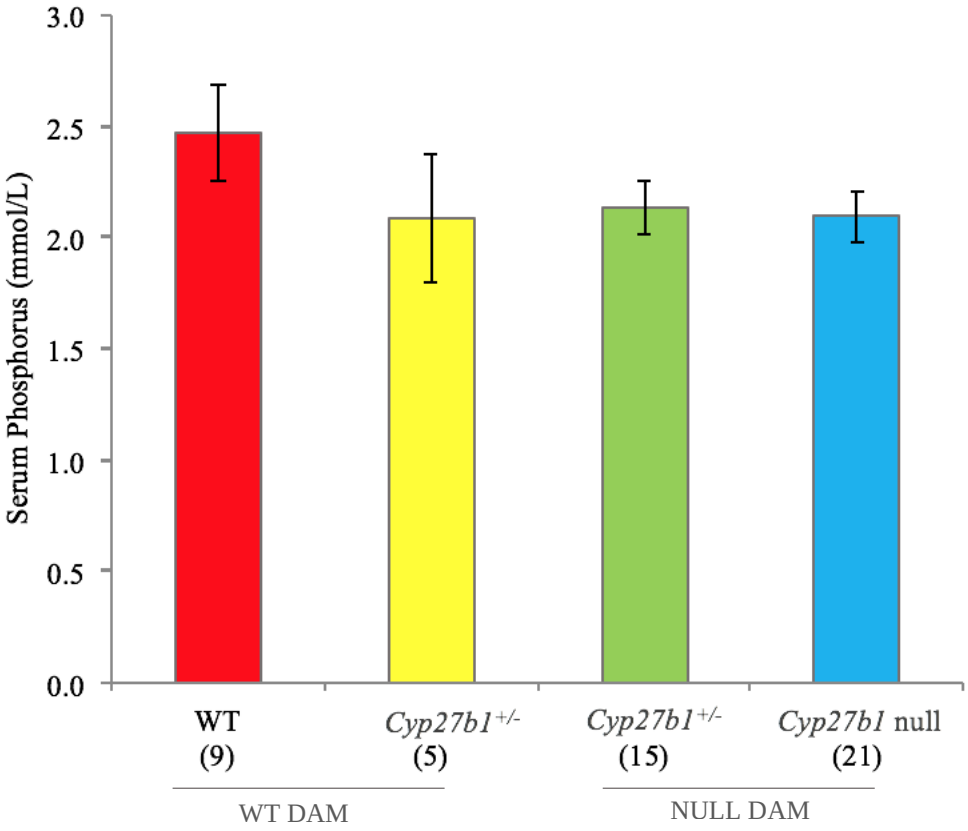

Figure 19: Serum Phosphorus in fetal mice borne of WT and *Cyp27b1* null mothers. Serum phosphorus is no difference between null and *Cyp27b1*$^{+/-}$ fetuses born of null mothers and WT and *Cyp27b1*$^{+/-}$ fetuses born of WT mothers. Values are means ± SE and the numbers of observations are indicated in parentheses.

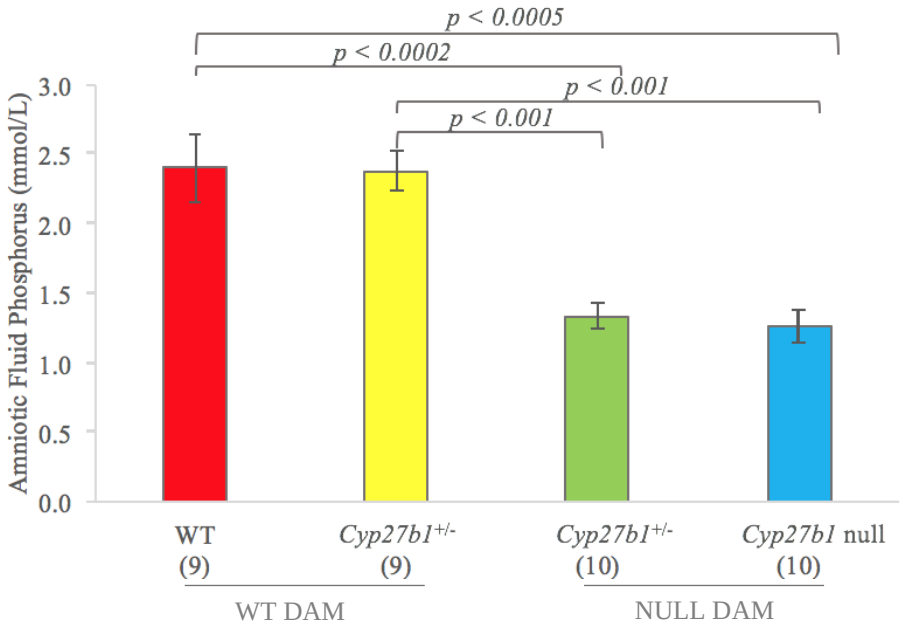

Figure 20: Amniotic fluid phosphorus in fetal mice borne of WT and *Cyp27b1* null mothers. Amniotic fluid phosphorus is significantly lower in fetuses of *Cyp27b1* null mothers when compared to fetuses of WT mothers. This suggests that maternal calcitriol plays a role in the regulation of fetal phosphorus excretion. Values are means ± SE and the numbers of observations are indicated in parentheses.

3.4 Fetal skeletal ash weight, mineral content and morphology

Ultimately circulating mineral ends up in the skeleton; therefore, if loss of calcitriol affects mineral transport or accretion of mineral into the bone, this should be visualized in the skeleton. Furthermore, if calcitriol is important for osteoblast or osteoclast function, then altered skeletal development may be a measurable outcome. To address this, fetal ash weight, mineral content and morphology were assessed.

3.4.1 Fetal ash weight and skeletal mineral content

Ash weight is a reflection of the total mineral content within the fetal skeleton. There was no difference in ash weight between null and *Cyp27b1*$^{+/-}$ fetuses of *Cyp27b1* null mothers (0.0151 ± 0.0008g vs. 0.0160 ± 0.0008g) or when compared to WT and *Cyp27b1*$^{+/-}$ fetuses of WT mothers (Figure 21).

There were no differences in ash calcium (Figure 22), phosphorus (Figure 23) or magnesium (Figure 24) content in *Cyp27b1* null fetuses compared to their *Cyp27b1*$^{+/-}$ littermates, or when compared to WT and *Cyp27b1*$^{+/-}$ littermates from WT mothers.

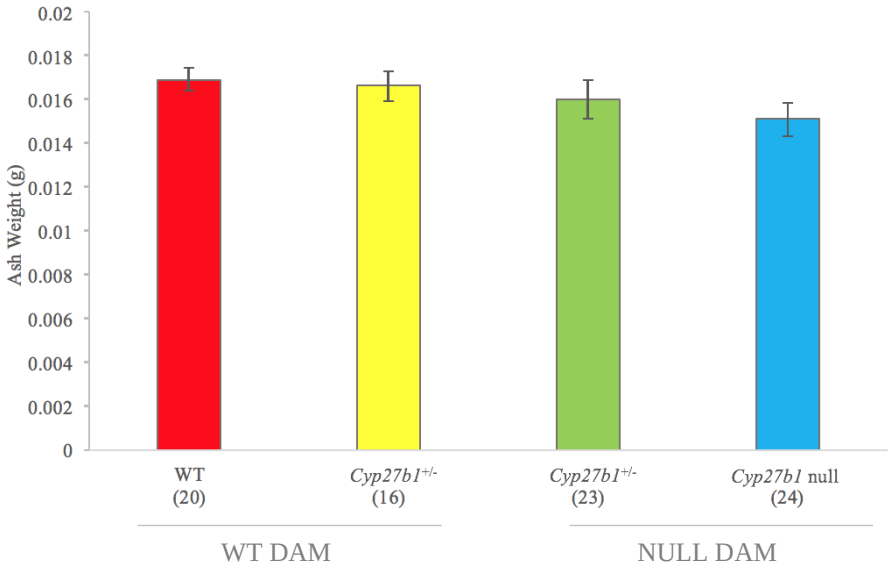

Figure 21: Ash Weight of fetal mice borne of WT and *Cyp27b1* null mothers. Fetal ash weight was no different between null and *Cyp27b1*$^{+/-}$ fetuses of *Cyp27b1* null mothers, or when compared to WT and *Cyp27b1*$^{+/-}$ fetuses of WT mothers. Values are means ± SE and the numbers of observations are indicated in parentheses.

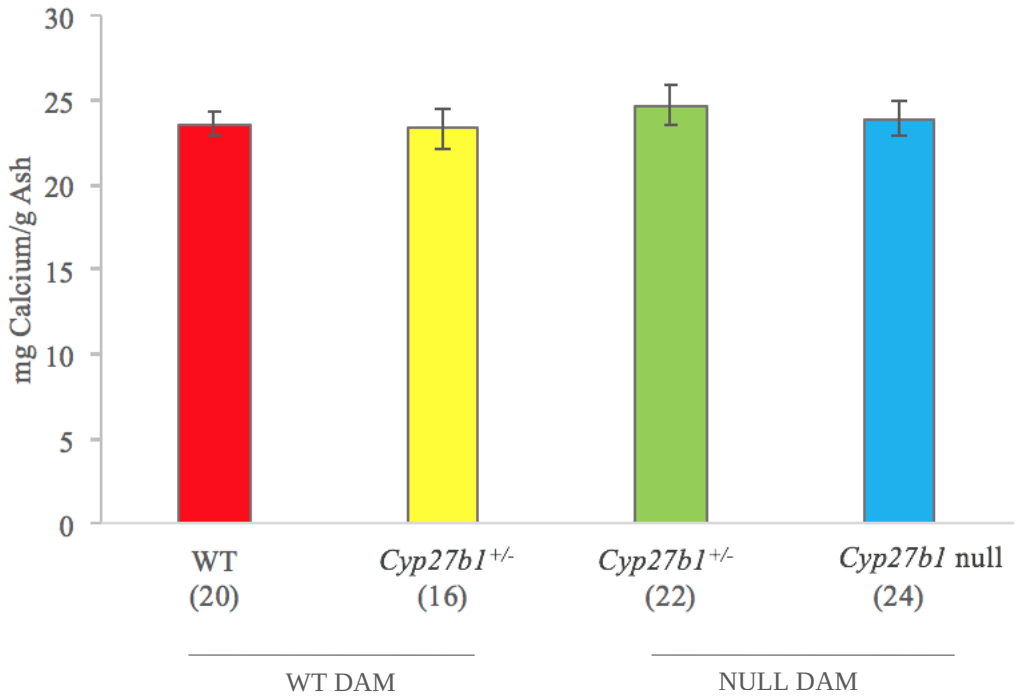

Figure 22: Skeletal calcium content in fetal mice borne of WT and *Cyp27b1* null mothers. Intact fetuses were reduced to ash and skeletal mineral content was measured using flame atomic spectrophotometry. Skeletal calcium content was no different between null and *Cyp27b1*$^{+/-}$ fetuses borne of *Cyp27b1* null mothers or when compared to WT and *Cyp27b1*$^{+/-}$ fetuses of WT mothers. Values are means ± SE and the numbers of observations are indicated in parentheses.

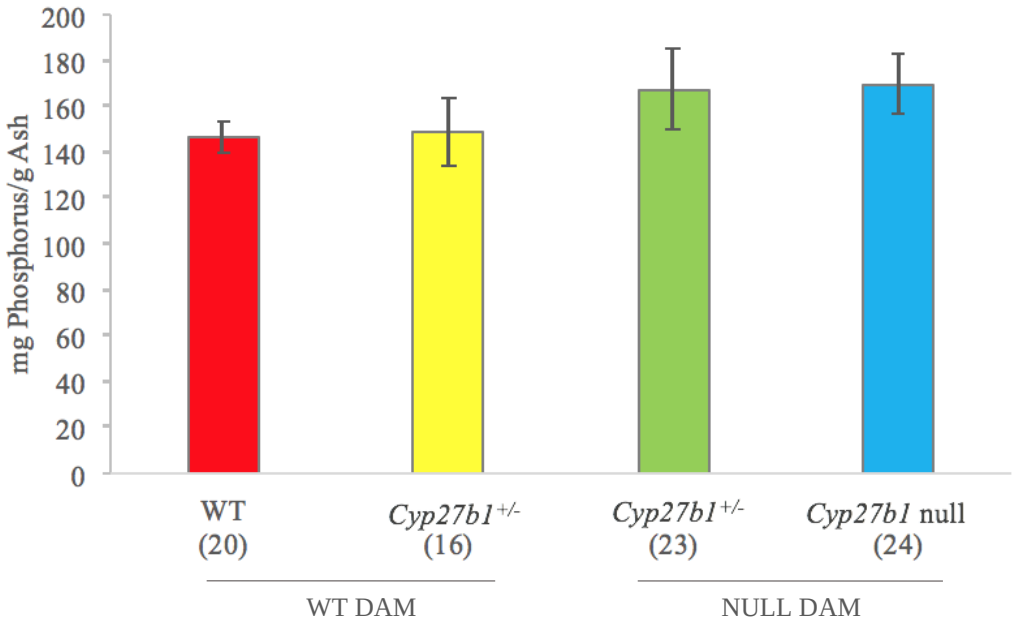

Figure 23: Skeletal phosphorus content of fetal mice borne of WT and *Cyp27b1* null mothers. Intact fetuses were reduced to ash and skeletal mineral content was measured using flame atomic spectrophotometry. Skeletal phosphorus content was no different between null and *Cyp27b1*$^{+/-}$ fetuses borne of *Cyp27b1* null mothers or when compared to WT and *Cyp27b1*$^{+/-}$ fetuses of WT mothers. Values are means ± SE and the numbers of observations are indicated in parentheses.

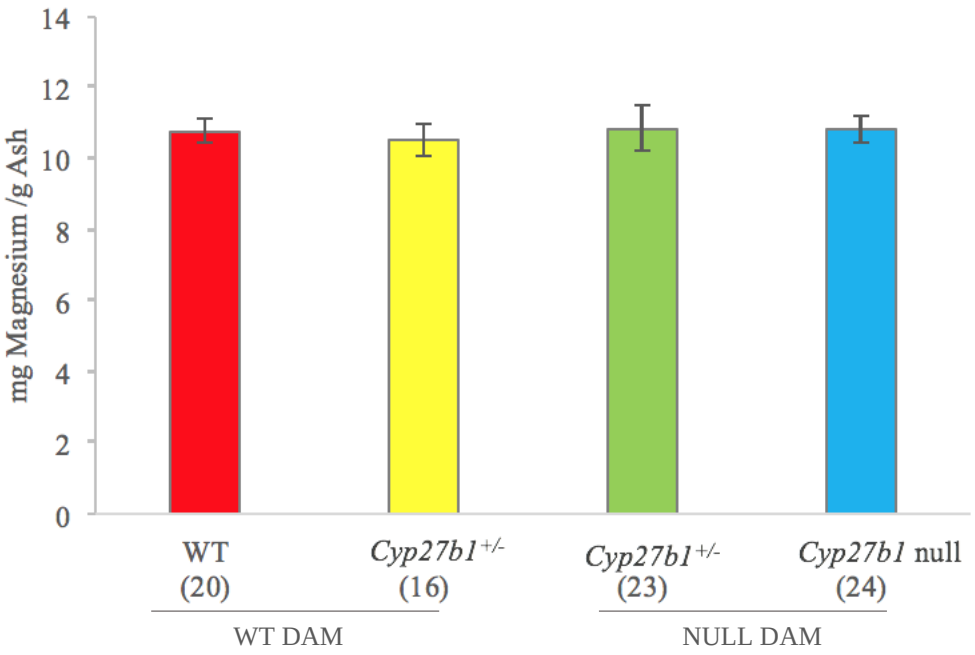

Figure 24: Skeletal magnesium content in fetal mice borne of WT and *Cyp27b1* null mothers. Intact fetuses were reduced to ash and skeletal mineral content was measured using flame atomic spectrophotometry. Skeletal magnesium content was no different between null and *Cyp27b1*$^{+/-}$ fetuses borne of *Cyp27b1* null mothers or when compared to WT and *Cyp27b1*$^{+/-}$ fetuses of WT mothers. Values are means ± SE and the numbers of observations are indicated in parentheses.

3.4.2 Tibial morphology

To examine skeletal development, the morphology of the fetal tibiae were examined, as it is the gold standard methodology recognized by the literature. The sequential development is visually laid out with the identifiable zones of chondrocytes within the growth plate followed by the start of the endochondral bone.

By use of this method, *Cyp27b1* null fetal tibias showed normal endochondral development with no differences in length or cellular morphology compared to tibial sections from WT fetuses born of WT mothers (Figure 25).

These results contrast with prior study of *Vdr* null fetuses which have shown increased placental calcium transport in the *Vdr* null fetuses compared to their $Vdr^{+/-}$ littermates.[131,137]

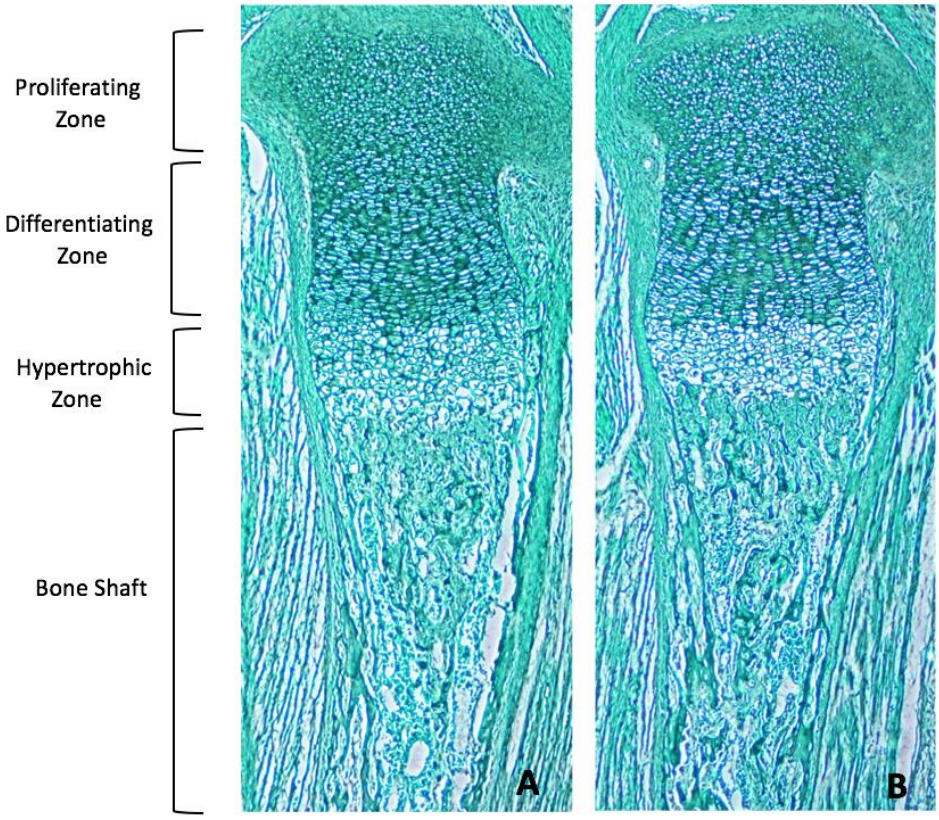

Figure 25: Fetal Tibial morphology. *Cyp27b1* null fetuses (B) show normal endochondral development, with no alteration in the length and cellular morphology of cartilaginous or boney compartments (as shown by methyl green stain), in comparison to WT fetuses borne of WT mothers (A). These images are representative of the individuals examined.

3.5 Placental calcium transport

Thus far, *Cyp27b1* null fetuses show no deficit in mineral concentrations in the circulation or accreted into the skeleton. We wanted to measure placental calcium transport to be certain that delivery of mineral to the fetus is also unaffected by absence of calcitriol, versus increased as it was in the *Vdr* nulls.

Placental calcium transport was not altered between *Cyp27b1* null and *Cyp27b1*$^{+/-}$ littermates or when compared to related *Cyp27b1*$^{+/-}$ and WT fetuses from WT mothers (Figure 26).

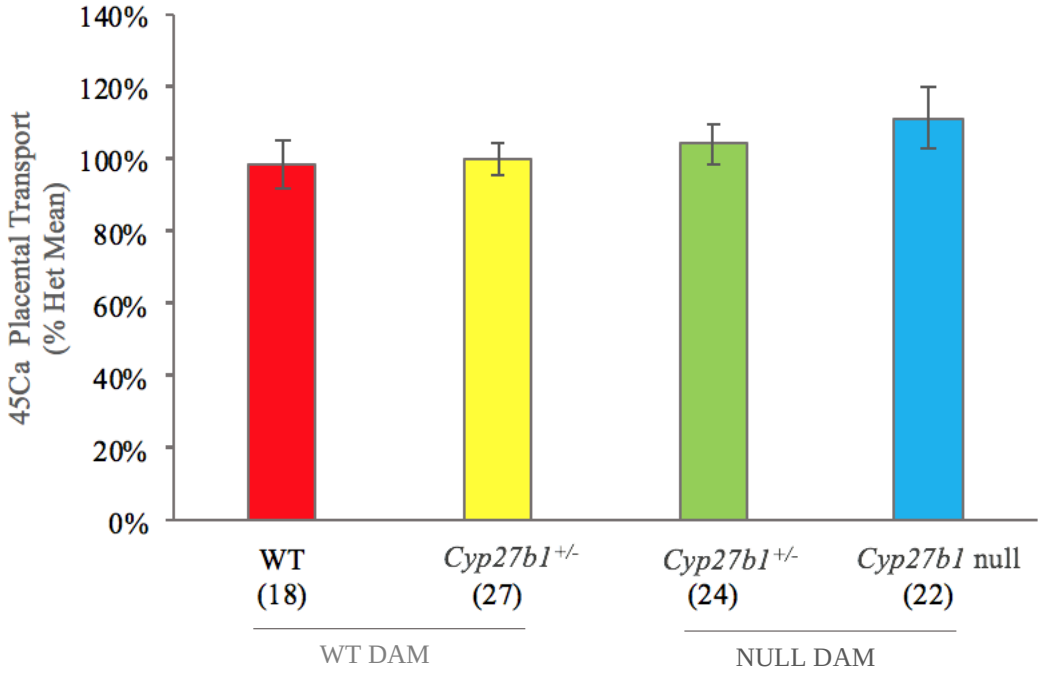

Figure 26: Placental calcium transport in fetuses borne of *Cyp27b1* null and WT mothers. Five minutes following injection of $^{45}Ca/^{51}Cr$-EDTA into the dam, *Cyp27b1* null fetuses showed the same amount of placental calcium transport as their *Cyp27b1*$^{+/-}$ littermates. Placental calcium transport was also no different when compared to WT and *Cyp27b1*$^{+/-}$ fetuses of WT mothers. Values are means ± SE and the numbers of observations are indicated in parentheses.

3.6 Expression of placental genes as assessed by qPCR

As there is normal placental calcium transport in *Cyp27b1* null fetuses, it seems as if placental function is normal despite loss of fetal calcitriol. However, we examined placental function further to determine if absence of calcitriol leads to any alteration in the expression of placental genes that are involved in calcium or phosphorus transport.

These genes were examined in placentas of *Cyp27b1* null vs. *Cyp27b1*$^{+/-}$ littermates, as well as in WT vs. *Cyp27b1*$^{+/-}$ littermates (Table 1). *Cyp27b1*$^{+/-}$ placentas showed robust expression of *Cyp27b1* which was absent in respective null placentas (1.000 ± 0.277 vs. 0.0068 ± 0.006). Separate comparison of *Cyp27b1* null vs. WT placentas showed absent expression of *Cyp27b1* in the null placentas.

There was no difference in expression of *Trpv6* (Figure 27AB) or *Pthrp* (Figure 28AB) between null and *Cyp27b1*$^{+/-}$ placentas, or between null and WT placentas.

Cyp24a1, Pmca1, S100g, Napi2a, Napi2b, Napi2c were all expressed in the placenta. There was a slight statistically significant decrease in expression of *Pmca1* in *Cyp27b1* null placentas, that may be physiologically insignificant. There were no other differences evident between *Cyp27b1* null and *Cyp27b1*$^{+/-}$ placentas, or between placentas of WT and Cyp27b1$^{+/-}$ littermates, for each of these genes (Table 1).

Low expression of *Fgf23* was present in *Cyp27b1* null and *Cyp27b1*$^{+/-}$ placenta (High C_T values of ~ 38 cycles). This corresponds with previous findings from our laboratory.

In contrast to the normal expression of *Trpv6* and *Pthrp* in *Cyp27b1* null placentas, previous study of *Vdr* null fetuses showed an upregulation in *Trpv6*[131,137] and *Pthrp*[131] in comparison to their littermates.

Table 1: Expression of genes related to calcium and phosphorus transport within the placenta of *Cyp27b1* null fetuses versus their *Cyp27b1*$^{+/-}$ siblings and WT fetuses versus their *Cyp27b1*$^{+/-}$ siblings. Values are means ± SD.

Gene	Fold change (*Cyp27b1* null vs. *Cyp27b1*$^{+/-}$littermates)	P value	Fold change (WT vs. *Cyp27b1*$^{+/-}$ littermates)	P value
Cyp27b1	0.007 ± 0.006 vs. 1.000 ± 0.277	P < 0.05	1.681 ± 0.307 vs. 1.000 ± 0.424	P <0.05
Cyp24a1	0.523 ± 0.476 vs. 1.000 ± 1.081	P=NS	1.353 ± 0.476 vs. 1.000 ± 0.613	P=NS
S100g	0.958 ± 0.358 vs. 1.000 ± 0.261	P=NS	0.967 ± 0.305 vs. 1.000 ± 0.241	P=NS
Pmca1	0.837 ± 0.079 vs. 1.000 ± 0.122	P < 0.05	0.873 ± 0.174 vs. 1.000 ± 0.076	P=NS
NaPi2a	0.861 ± 0.679 vs. 1.000 ± 0.442	P=NS	1.404 ± 0.620 vs. 1.000 ± 0.428	P=NS
NaPi2b	1.151 ± 0.637 vs. 1.000 ± 0.317	P=NS	0.693 ± 0.275 vs. 1.000 ± 0.327	P=NS
NaPi2c	0.841 ± 0.731 vs. 1.000 ± 0.724	P=NS	0.927 ± 0.276 vs. 1.0007 vs. 0.276	P=NS

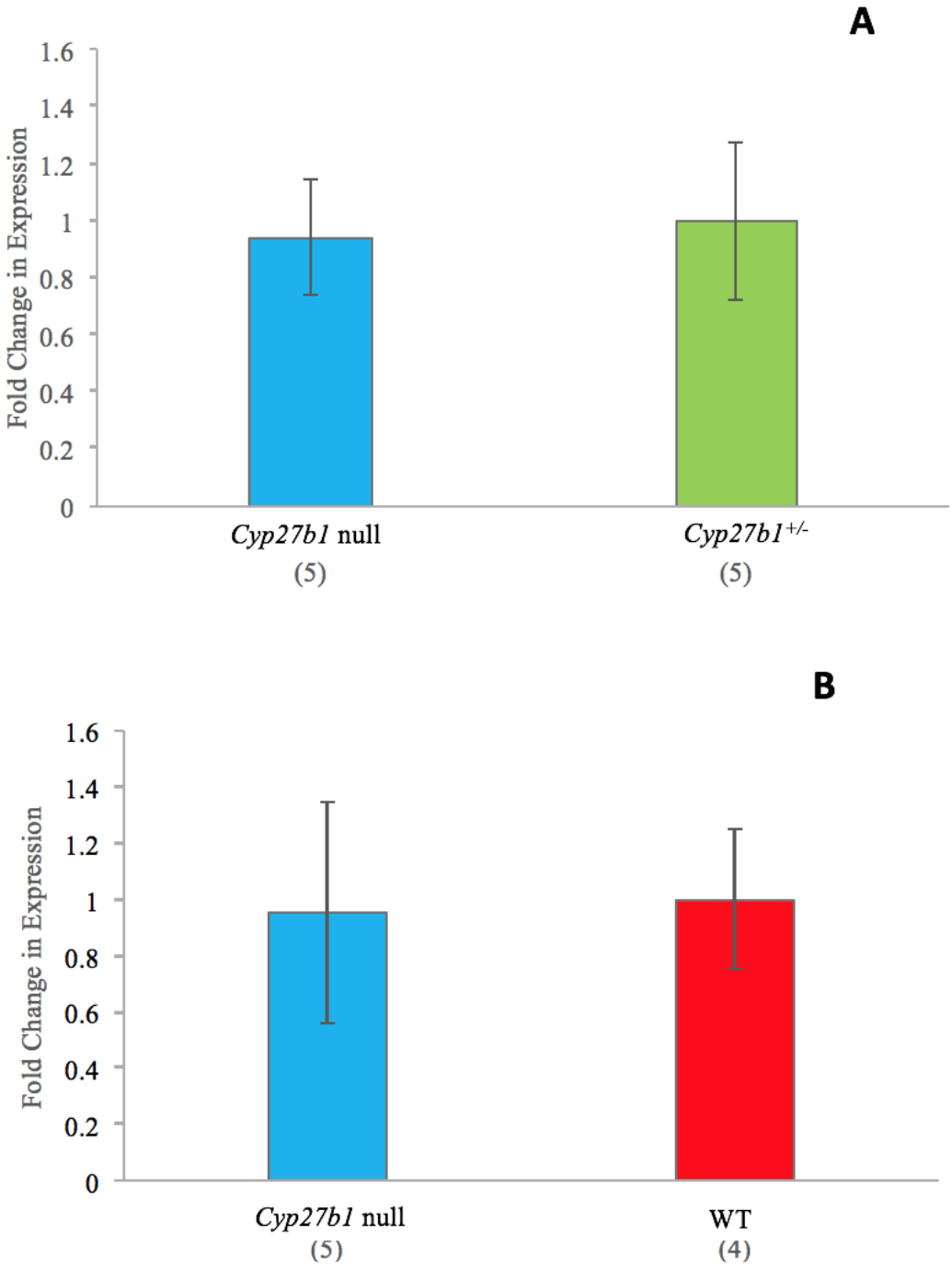

Figure 27: Expression of *Trpv6* mRNA in *Cyp27b1* null versus *Cyp27b1*$^{+/-}$ placentas (A) and in *Cyp27b1* null versus WT placentas (B). Values are means ± SD and the number of observations are indicated in parentheses.

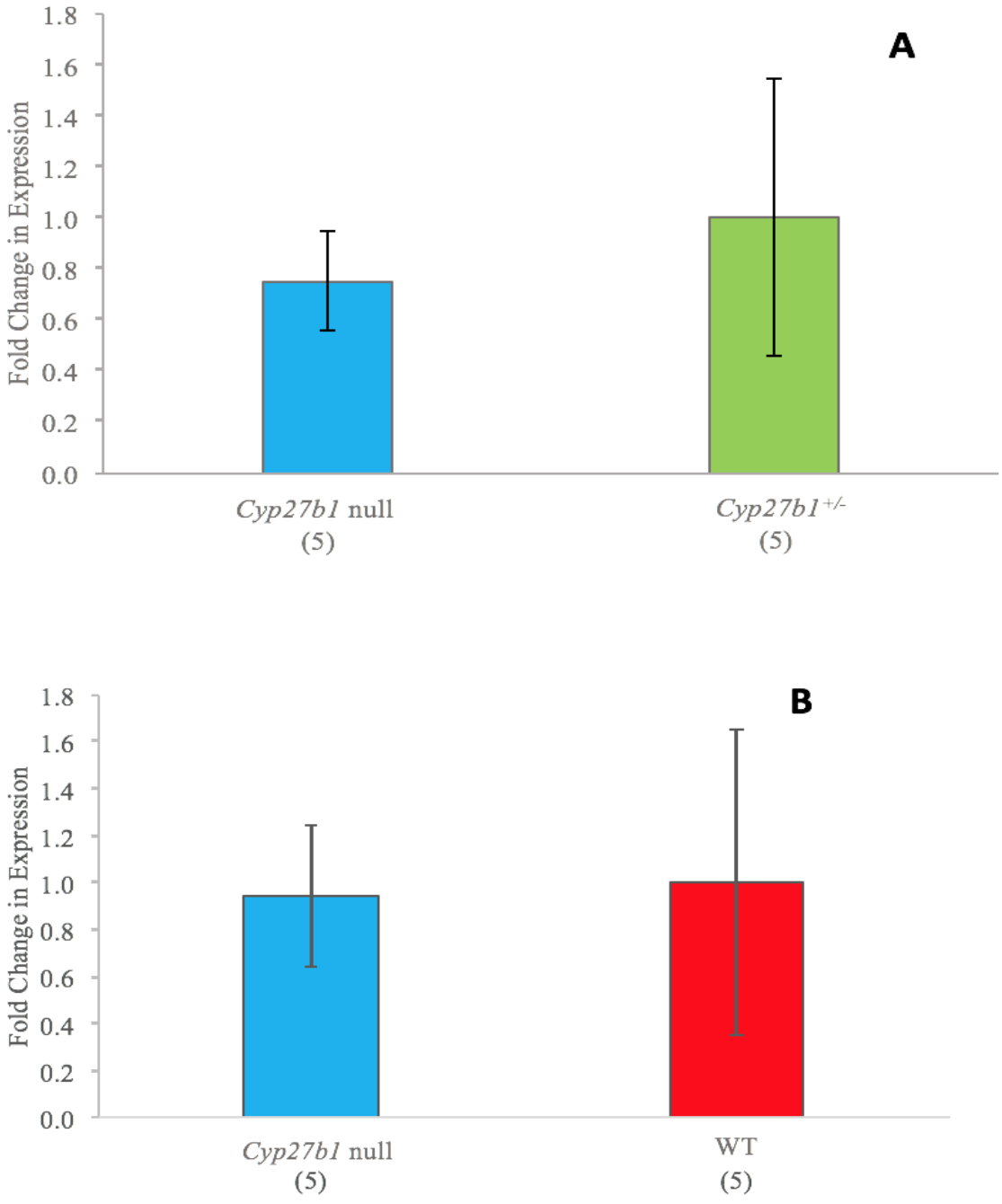

Figure 28: Expression of *Pthrp* in *Cyp27b1* null versus *Cyp27b1*$^{+/-}$ placentas (A) and in *Cyp27b1* null versus WT placentas. Values are means ± SD and the number of observations are indicated in parentheses.

3.7 Expression of renal genes as assessed by qPCR

Expression of genes related to calcium and phosphorus transport were examined in kidneys of *Cyp27b1* null fetuses and their *Cyp27b1*$^{+/-}$ littermates, as well as in kidneys of WT fetuses and respective *Cyp27b1*$^{+/-}$ littermates (Table 2).

Cyp27b1 null fetal kidneys showed the expected absent expression of *Cyp27b1*. In a comparison of WT kidneys versus their *Cyp27b1*$^{+/-}$littermates, there appeared to be a trend towards higher *Cyp27b1* expression in the WT kidneys; however, this was not statistically significant (Table 2).

Between *Cyp27b1* null fetuses and *Cyp27b1*$^{+/-}$ littermates there were no differences in expression of *Cyp24a1*, calcium transporter *Pmca1* or *Napi2a*, *Napi2b* and *Napi2c*.

We did observe a significant decrease in renal expression of *S100G*, which encodes for Calbindin-D_{9k}, between null fetuses and *Cyp27b1*$^{+/-}$ littermates (0.304±0.11 vs. 1.00±0.63). This data is consistent with the role of Calbindin-D_{9k} in calcium transport in the kidney.

Table 2: Expression of genes related to calcium and phosphorus transport within the kidney of *Cyp27b1* null fetuses versus their *Cyp27b1*$^{+/-}$ siblings and WT fetuses versus their *Cyp27b1*$^{+/-}$ siblings. Values are means ± SD.

Gene	Fold Change (*Cyp27b1* null vs. *Cyp27b1*$^{+/-}$ littermates)	P Value	Fold Change (WT vs. Cyp27b1$^{+/-}$ littermates)	P Value
Cyp27b1	Absent Expression	N/A	1.408 ± 0.364 vs. 1.000 ± 0.239	P = NS
Cyp24a1	1.252 ± 1.232 vs. 1.000 ± 0.576	P = NS	1.221 ± 0.563 vs. 1.000 ± 0.614	P = NS
Pmcab1	1.038 ± 0.142 vs. 1.000 ± 0.265	P = NS	0.987 ± 0.158 vs. 1.000 ± 0.212	P = NS
S100g	0.304 ± 0.114 vs. 1.000 ± 0.626	P < 0.05	0.823 ± 0.110 vs. 1.000 ± 0.341	P = NS
Napi2a	0.847 ± 0.436 vs. 1.000 ± 0.386	P = NS	0.865 ± 0.214 vs. 1.000 ± 0.276	P = NS
Napi2b	1.059 ± 0.251 vs. 1.000 ± 0.329	P = NS	0.959 ± 0.246 vs. 1.000 ± 0.082	P = NS
Napi2c	0.866 ± 0.271 vs. 1.000 ± 0.436	P = NS	0.830 ± 0.218 vs. 1.000 ± 0.527	P = NS

IV. DISCUSSION

The role of calcitriol, the active, hormonal form of vitamin D, is critically important in the regulation of calcium and bone metabolism in both the child and the adult.[23,24,159] Without calcitriol's actions to stimulate passive and active absorption of calcium and phosphorus, serum calcium and phosphorus are low, PTH levels are increased, while bone grows abnormally and is undermineralized. However, the role of calcitriol in fetal bone and mineral homeostasis is less clear.

Prior study of fetuses with disrupted vitamin D physiology, including vitamin D deficient rodents, *Cyp27b1* null pigs, and *Vdr* null fetuses, have suggested that calcitriol is not required to regulate fetal bone and mineral metabolism. In particular, *Vdr* null fetuses, which have the genetic inability to respond to calcitriol, have normal serum calcium, phosphorus, PTH, amniotic mineral content, skeletal morphology, and skeletal mineral content in comparison to WT siblings.[131,137] Furthermore, calcitriol must not be required to stimulate placental calcium transport because *Vdr* null fetuses were found to have an increased rate of transport, and upregulated placental expression of two factors that are required for placental calcium transport: PTHrP and the calcium channel TRPV6. These findings were consistent with the vitamin D deficiency models, and suggested that calcitriol is not required to regulate fetal calcium homeostasis, skeletal development or mineralization, and placental mineral transfer. However, *Vdr* null fetal mice were found to have high circulating levels of calcitriol.[131,137] Therefore, we hypothesized first that calcitriol is not required for bone and mineral homeostasis. Secondly, we hypothesized that the upregulation of placental function in the *Vdr* null fetuses may be due to calcitriol

having physiological effects to reduce placental calcium transfer, such that when calcitriol has no receptor to signal through, calcium transport increases due to loss of calcitriol's normal effects as a "brake" on the forward flow. However, this hypothesis is unable to explain why models of vitamin D deficiency do not show increased placental calcium transport.[169] Alternatively, we considered that since *Vdr* null fetuses have higher than normal concentrations of calcitriol, it is conceivable that such high levels of calcitriol act through non-classical receptors, such as Pdia3 (discussed below) to upregulate placental calcium transport despite the absence of VDR. Such an action might not be physiological but only brought about with high concentrations of calcitriol, and this would explain why both *Vdr* null fetal mice and fetal sheep treated with pharmacological doses of calcitriol had increased placental calcium transport.

In order to test these hypotheses, we studied *Cyp27b1* null fetuses, who cannot synthesize calcitriol. If our first hypothesis held true, *Cyp27b1* null fetuses would have normal serum minerals, hormones, bone mineral and bone morphology in comparison to their *Cyp27b1*$^{+/-}$ littermates and related WT fetuses. If our second hypothesis held true then placental function would be upregulated in *Cyp27b1* null fetuses. However, if the third hypothesis held true, placental function would be normal, or possibly downregulated in the *Cyp27b1* null fetuses.

In brief, our main findings were that *Cyp27b1* null fetuses have normal placental calcium transport and expression of genes related to placental calcium transport, *Trpv6* and *Pthrp*,

which supports that the third hypothesis is confirmed by the data. I will now discuss these results in more detail.

4.1 Maternal calcitriol can rescue Cyp27b1 null fetuses

Previous research from our lab (unpublished) set out to examine *Cyp27b1* null fetuses using a traditional breeding scheme, by mating *Cyp27b1*$^{+/-}$ males and females together to generate mixed sex litters of null, heterozygous and WT offspring.[160] *Cyp27b1* null fetuses derived from these crosses were determined to have normal serum calcium, phosphorus, PTH, amniotic mineral content and skeletal morphology in comparison to their WT siblings. However, this approach was confounded by the unexpected finding of detectable amounts of calcitriol in the null fetuses, due to passage of calcitriol across the placenta from the *Cyp27b1*$^{+/-}$ mothers. Most previously published data had suggested that 25OHD crosses the placenta, whereas vitamin D and calcitriol do not.[93] However, there is one study that supports the passage of calcitriol across the placenta.[162] Therefore, while this breeding approach did not result in a true calcitriol-depleted fetus, it confirmed the presence of significant passage of calcitriol across the placenta from the maternal circulation. Moreover, it suggests that infants lacking *Cyp27b1* have calcitriol at birth due to "rescue" from their heterozygous mothers. The current project compared fetuses from *Cyp27b1* null mothers to those of WT mothers. In this way *Cyp27b1* null fetuses were truly calcitriol deficient.

4.1.1 Cyp27b1 null fetuses of Cyp27b1 null mothers are devoid of calcitriol, although Cyp27b1 null mothers gain calcitriol from their Cyp27b1$^{+/-}$ fetuses

Calcitriol was undetectable in *Cyp27b1* null fetuses, and equivalent to the values observed in non-pregnant *Cyp27b1* null mice. Thus, *Cyp27b1* null fetuses generated in this manner truly lacked calcitriol. On the other hand, the calcitriol level of *Cyp27b1* null pregnant mothers appeared to increase compared to the non-pregnant value. This suggests that there was reverse flow of calcitriol across the placenta from heterozygous placentas or fetuses to the mother, thereby partly rescuing the mother. We have reported this previously in our studies of maternal physiology of *Cyp27b1* null females across pregnancy and lactation.[159] This phenomenon has also been shown in studies of anephric rats administered radio-labelled 25OHD. In these studies, some calcitriol was evident in pregnant dams while non-pregnant rats had no calcitriol.[163,164] This suggests that the placenta can contribute some calcitriol to the maternal circulation in rodent models. The same cannot be said for humans, as demonstrated through a vitamin D sufficient, anephric pregnant woman who continued to have low calcitriol levels despite her baby's normal placenta.[165]

However, although the *Cyp27b1* null mothers had some calcitriol, this was evidently not enough to rescue their *Cyp27b1* null fetuses, since their calcitriol level was undetectable. Of course, the possibility that what was undetectable by ELISA might have been detectable by the standard methodology of liquid chromatography tandem mass spectrometry (LC-MS/MS) cannot be fully eliminated. Regardless, it is clear that *Cyp27b1* null fetuses had either no calcitriol or very low levels of it, and were largely

unaffected by its absence.

4.2 Calcitriol is not required to regulate fetal bone and mineral homeostasis

A number of experiments were conducted in order to assess calcitriol's role in the regulation of fetal bone and mineral homeostasis. In depth study of *Cyp27b1* null fetuses borne of *Cyp27b1* null mothers demonstrated no differences in serum calcium, phosphorus, PTH, ash weight and skeletal mineral content, tibial morphology, placental gene expression or placental calcium transport. These results support our hypothesis that calcitriol is not required to regulate fetal bone and mineral homeostasis. If calcitriol were required, we would have expected to see decreased serum calcium and phosphorus, an increase in PTH, as well as rachitic changes in the tibias.

Prior animal data are consistent with these findings. Study of vitamin D deficiency, loss of VDR, and loss of *Cyp27b1,* have all indicated that calcitriol is not required to maintain serum calcium and phosphorus, PTH, ash weight, skeletal mineral content, skeletal morphology and transport of calcium and phosphorus across the placenta.[93] There are two reasons that may explain why calcitriol is not required in the regulation of fetal bone and mineral metabolism. Firstly, it is clear that calcitriol's main role in both the child and the adult is to stimulate intestinal calcium absorption,[21] as deletion of VDR from only the intestinal cells causes a rachitic phenotype in mice, while *Vdr* null mice can be genetically rescued (phenotypically normal) by selectively expressing VDR only within intestinal cells.[73,74] However, within the fetal system, the intestinal mineral absorption

pathway is a minor circuit as the placenta is the dominant organ of mineral transport from mother to fetus.[121,147,148] Secondly, calcitriol is not required for mineral transport across the placenta to occur, [131,139] since the absence of calcitriol in *Cyp27b1* null mice did not reduce or increase placental calcium transport.

4.3 Differences in loss of *Cyp27b1* and VDR on placental function

Although study of vitamin D deficient, *Vdr* null and *Cyp27b1* null fetuses have shown clear and consistent evidence that calcitriol does not play a role in fetal bone and mineral homeostasis, there are notable differences between *Vdr* null and *Cyp27b1* null placentas.

Prior study from our lab of *Vdr* null fetuses, which have high levels of circulating calcitriol, indicated a non-significant increase in placental calcium transport in both *Vdr* null fetuses born of $Vdr^{+/-}$ mothers and those born of *Vdr* null mothers in comparison to WT littermates, when studied in isolation. However, when these data sets were pooled, a significant increase in placental calcium transport in the Vdr null fetuses was revealed. *Vdr* null fetuses also displayed an increased expression of placental *Pthrp*, known to stimulate placental calcium transport, and *Trpv6* mRNA[131] These findings were later confirmed by an independent group using an alternate *Vdr* null model, which also displayed significant increases in placental calcium transport, and placental expression of calcium transporter *Trpv6* mRNA in *Vdr* null fetuses in comparison to their $Vdr^{+/-}$ littermates.[137]

In contrast, in the current study, *Cyp27b1* null fetuses, which cannot synthesize calcitriol, had normal transport of calcium across the placenta, as well as normal placental expression of both *Trpv6* and *Pthrp* mRNA, as compared to both their *Cyp27b1*$^{+/-}$ littermates and related WT fetuses.

Taken together, these comparative results imply that the high levels of circulating calcitriol in the *Vdr* null fetuses are able to act on an alternate receptor to stimulate placental calcium transport. This may explain why some prior animal studies found an increase in placental calcium transport in animals treated with pharmacological doses of calcitriol.[93] However, at normal physiological concentrations, calcitriol may have no significant effect on placental calcium transport or the placental expression of *Pthrp* and *Trpv6*, as loss of *Cyp27b1* does not alter these placental parameters. This is consistent with vitamin D deficiency models which exhibit normal levels of placental calcium transport. [139,140] Overall, this may imply that the "pharmacological" effects of very high calcitriol concentrations in *Vdr* null fetuses, and with pharmacological administration of calcitriol in normal fetuses, may not be relevant to normal physiology. However, these findings may still be relevant to human pregnancies in which the baby has HVDRR, related to loss of VDR signaling.

4.5 Renal response to calcitriol

Renal gene expression studies indicated that loss of fetal *Cyp27b1* had no effect on expression of calcium transporter Ca^{2+}- ATPase or the sodium phosphate transporters

Napi2a, Napi2b and *Napi2c*. However, there was an observed decrease in calcium transporter *S100G*, which encodes for calcium binding protein Calbindin-D_{9k}, in *Cyp27b1* null fetuses in comparison to their *Cyp27b1*$^{+/-}$ littermates. This result confirms that the kidneys are responsive to fetal calcitriol levels. The decrease in renal expression of *S100G* in the *Cyp27b1* null fetuses is consistent with calcitriol's ability to stimulate Calbindin-D_{9k} to transport calcium within the kidney cells. The absence of an effect due to fetal calcitriol on renal excretion of calcium and phosphorus (as measured by amniotic fluid mineral content), adds to previous evidence that the renal-amniotic fluid pathway plays a minimal role in fetal mineral homeostasis. These data contrast with placental expression of *S100G*, which was unchanged in *Cyp27b1* null fetuses in comparison to their *Cyp27b1*$^{+/-}$ littermates. The lack of change in placental *S100G* is consistent with prior studies in vitamin D deficiency models,[139,140] and suggests that *S100G* is not regulated by calcitriol in the placenta.

4.6 Maternal calcitriol directly or indirectly regulates fetal mineral metabolism

Although the presented evidence is that fetal calcitriol does not regulate fetal bone and mineral homeostasis, maternal loss of *Cyp27b1*, and therefore calcitriol, had modest effects on the fetuses that were independent of fetal genotype. Whether these were direct or indirect effects is not clear. Lack of maternal *Cyp27b1* led to significantly increased concentration of calcitriol's precursor $25OHD_3$ and the vitamin D metabolite 24,25-dihydroxyvitamin D in the fetal serum. It is likely that lack of maternal metabolism of $25OHD_3$ into calcitriol in the *Cyp27b1* null mothers led to higher values crossing the

placenta where it accumulates to reach a higher concentration of $25OHD_3$. Consequently, the higher amount of $25OHD_3$ may have led to more 24,25-dihydroxyvitamin D_3 simply due to more substrate being present. Fetuses borne of *Cyp27b1* null mothers also had increased calcium content and decreased phosphorus content in amniotic fluid (an index of fetal kidney function), and significantly reduced serum FGF23. PTH was non-significantly decreased in fetuses of the *Cyp27b1* null mothers, as compared to those borne of WT mothers. In each of these cases, both *Cyp27b1* null and *Cyp27b1*$^{+/-}$ fetuses borne of *Cyp27b1* null mothers were indistinguishable, but different from both *Cyp27b1*$^{+/-}$ and WT fetuses borne of WT dams. This suggests that these differences are solely due to loss of maternal calcitriol, and not due to fetal genotype. As there are differences due to maternal genotype across multiple parameters, it is convincing that maternal genotype has a real effect on fetal parameters that are independent of fetal genotype. It is currently unknown whether these differences lead to any persistent changes in the neonates or older pups.

These results indicate that maternal calcitriol plays a direct or indirect role in regulating fetal FGF23 and fetal renal excretion of calcium and phosphorus. Also, by implication, fetal PTH, since the non-significantly lower PTH levels in fetuses of null dams might explain the findings of increased calcium and reduced phosphorus in amniotic fluid. It is likely that maternal calcitriol has a direct effect on these parameters of fetal bone and mineral homeostasis, as preliminary work in our lab which led to my project showed detectable levels of calcitriol in *Cyp27b1* null fetuses borne of *Cyp27b1*$^{+/-}$ mothers.[160] This is supported by a prior study of human placenta and an in vitro perfusion system to

determine that calcitriol can cross the placenta.[162] Furthermore, it is known that 25OHD readily crosses the placenta, so it is logical that calcitriol would also be able to cross the placenta as it only differs by one hydroxyl group. [93]

4.7 Rescue Diet

Supplementation with calcium, phosphorus and lactose has been shown to prevent the rachitic phenotype and normalize fertility in *Cyp27b1* null and *Vdr* null post-weaning and adult mice.[155,166] Similar findings have also been demonstrated in human studies, in which the lactose content of breast milk has been shown to increase calcium absorption in babies.[166] The ability of the rescue diet to prevent the rachitic phenotype is through passive intestinal calcium absorption and is consistent with the important roles of both calcitriol and VDR on intestinal calcium and phosphorus absorption through upregulation of calcium transporters.[26,141,168] This may help explain why children and adults with vitamin D deficiency display a rachitic phenotype but fetuses do not. The placenta is the dominant supplier of mineral in the fetal circulation and the intestines play a much lesser role. Neither calcitriol nor VDR are required for placental mineral transport.[131]

Thus, for the purpose of this study, and to ensure that fertility was optimized, *Cyp27b1* null and WT mothers were kept on an enriched rescue diet (2% calcium, 1.25% phosphorus and 20% lactose) in order to support fertility.

4.8 Study Limitations

4.8.1 Comparison of fetuses borne of Cyp27b1 null versus WT mothers

As previously mentioned, prior study in our lab determined that *Cyp27b1* null fetuses borne of *Cyp27b1*$^{+/-}$ mothers have detectable levels of calcitriol due to significant transplacental passage of calcitriol from the mother. Thus, the C*yp27b1* null fetuses generated for this study came from matings of *Cyp27b1* null females with *Cyp27b1*$^{+/-}$ males. While this mating scheme was successful in generating true calcitriol-depleted fetuses, it doesn't allow for null and WT fetuses to be compared within the same litter. However, this may be considered a strength in that WT and *Cyp27b1* null mothers are close relatives from the same colony and we were able to control for any changes that may be displayed in the fetuses due to maternal differences by comparing *Cyp27b1*$^{+/-}$ fetuses borne of *Cyp27b1* null and WT mothers. This allowed us to determine that the significant differences in serum FGF23, amniotic fluid calcium and amniotic fluid phosphorus content, between *Cyp27b1* null and WT fetuses were due to loss of maternal calcitriol rather than loss of fetal calcitriol. It is possible to generate WT and null fetuses within the same uterus; however, it would require transferring blastocysts in culture to pseudopregnant dams, which is well-beyond the scope of this project.

4.8.2 Use of the rescue diet

Use of the rescue diet which is high in calcium, phosphorus and lactose, may confound our findings by providing extra mineral to the *Cyp27b1* null mothers. However, the use of the rescue diet is necessary in order to allow for normal fertility, and thereby provide readily available pregnancies to study *Cyp27b1* null fetuses during the time frame of a

Master's project. Moreover, use of the rescue diet mimics the human condition, because pregnant women lacking *CYP27B1* are treated with high doses of calcium,[93] if they are unable to afford calcitriol treatment which is quite expensive. To control for any differences due to rescue diet, both WT and *Cyp27b1* null mice were kept on the rescue diet from birth.

4.8.3 Calcitriol assay

There is a possibility that there were circulating levels of calcitriol in the *Cyp27b1* null fetuses that were not detected by the ELISA assay. However, Dr. Glenville Jones, a expert in the field in vitamin D measurement, suggests that the ELISA assay is more sensitive than the standard methodology of LC-MS/MS (personal correspondence). Therefore, calcitriol levels would have also been undetectable using this LC-MS/MS.

4.8.4 The ability of the mouse model to reflect the human condition

The *Cyp27b1* mouse model is used to study PDDR, characterized by loss of function of the *CYP27B1*gene in humans. The *Cyp27b1* knockout mouse model mirrors PDDR quite well. Much like humans with PDDR, who appear normal at birth and present with symptoms of rickets at 1-2 years of age, *Cyp27b1* null mice also appear normal at birth and present with hypocalcemia, hypophosphatemia, secondary hyperparathyroidism and rickets after weaning. [81] One notable difference between the *Cyp27b1* mouse model and the human condition is that calcitriol is able to pass through the placenta from the fetus to the maternal circulation in the mouse model, whereas the human placenta does not allow for this passage of calcitriol from the fetus to the mother. [165]

4.9 Relevance to Human Health

The *Cyp27b1* mouse model is very consistent with the human condition of PDDR. Moreover, such studies are not possible on human fetuses, so we must rely on the animal models to inform us on the human condition. Although data from these studies suggest that calcitriol is not required for fetal bone and mineral homeostasis, they do not imply that vitamin D insufficiency should be ignored during human pregnancy. Instead, they reassure us that human fetuses born of vitamin D deficient mothers, as well as mothers with PDDR or HVDRR, should be normal, as previous clinical studies have also suggested. [169] In addition, we know that the neonate needs vitamin D shortly after birth (as hypocalcemia can begin as early as 48 hours after birth in fetuses born of vitamin D deficient mothers), and so it is best that the newborn start off with sufficient levels, even if unnecessary during fetal life.

4.10 Future work

4.10.1 Identification of calcitriol's alternate putative receptor

Contrasting data of placental function between *Vdr* null fetuses (which have high levels of circulating calcitriol) and *Cyp27b1* null fetuses (which cannot synthesize calcitriol) suggest that calcitriol may act on an alternate receptor, that has not yet been identified, in order to upregulate placenta calcium transport and related gene expression in the *Vdr* null fetuses. A future direction for this project includes identification of this alternate putative receptor. RNA-sequencing of placental tissue may help to identify the differentially

regulated genes that allow calcitriol to upregulate placental function in the *Vdr* null fetuses. One possible known receptor to study is Pdia3, which is associated with rapid membrane-initiated signaling by calcitriol.[170] In order to study this, we would ideally generate a double knockout of *Vdr/Pdia3.* However, since previous study of *Pdia3* null mice have shown that *Pdia3* null fetuses die before ED 12,[170] it would be best to study mice that are both *Vdr null* and have a conditional (floxed) knockout of *Pdia3* within their placentas. An alternative approach to this would be to cross *Vdr* null mice with *Pdia3* heterozygotes. Pdia3 heterozygotes have a distinctive bone phenotype which suggests that this may be a possible solution.[170]

4.10.2 Confirmation of calcitriol's role in upregulation of placental calcium transport in Vdr null fetuses

Since these studies found that loss of calcitriol, through loss of *Cyp27b1*, did not appreciably disturb normal placental function, it appears that the high levels of circulating calcitriol in *Vdr* null fetuses are able to work on an alternate receptor in order to upregulate placental calcium transport. To confirm this, *Cyp27b1/Vdr* double knockout mice could be studied.[171] In this way, if *Cyp27b1/Vdr* double knockout fetuses have normal placental calcium transport, we would know for sure that it is the high levels of calcitriol in the *Vdr* null fetuses that upregulate placental calcium transport in these mice. Alternatively, this could be studied using mice that are both *Vdr* null and vitamin D deficient.

4.10.3 Postnatal study of *Cyp27b1* null pups

To date, there are no studies that look at the role of calcitriol in the neonate. It would be interesting to study *Cyp27b1* null pups borne of *Cyp27b1* null mothers after weaning to determine if the effects due to loss of maternal calcitriol that were evident *in utero* lead to any progressive alterations in mineral or bone physiology in the offspring before or after weaning.

4.10.4 Study of *Cyp27b1* null mothers mated to *Cyp27b1* null males

It would be ideal to conduct an experiment to compare *Cyp27b1* null mothers mated to *Cyp27b1* null males with *Cyp27b1* null mothers mated to *Cyp27b1*$^{+/-}$ males, to be certain that the calcitriol in the mother in the current study isn't rescuing the null fetuses. But this is beyond the scope of the current project, but may pose an interesting follow-up study.

4.11 Summary

Transplacental passage of calcitriol from *Cyp27b1*$^{+/-}$ mothers to their fetuses can provide near-normal concentrations of calcitriol in fetuses lacking *Cyp27b1*. However, when *Cyp27b1* null fetuses are borne of *Cyp27b1* null mothers, and thus are truly calcitriol depleted, they display normal serum minerals, PTH, FGF23, renal excretion of calcium and phosphorus into the amniotic fluid, placental calcium transport, as well as placental and renal expression of genes related to calcium and phosphorus transport. Of importance, the normal placental calcium transport and placental expression of *Trpv6* and *Pthrp* mRNA contrasts that of *Vdr* null fetuses, which have increased placental calcium transport, and expression of *Trpv6* and *Pthrp* mRNA in comparison to their *Vdr*$^{+/-}$ and

WT counterparts.[131,137] These results suggest that high or pharmacological levels of calcitriol may act on an alternate receptor to stimulate placental calcium transport and expression of *Trpv6* and *Pthrp*. Such actions of calcitriol are not necessarily physiological, since WT fetuses normally have low levels of calcitriol. Furthermore, fetuses borne of *Cyp27b1* null mothers differ in serum 25OHD, 24,25-dihydroxyvitamin D, FGF23, and amniotic fluid calcium and phosphorus, and possibly PTH, in comparison to fetuses borne of WT mothers. These findings suggest that maternal calcitriol has effects on fetal mineral homeostasis that are independent of the fetal genotype. Whether they are direct or indirect effects of maternal calcitriol will be determined in future studies.

4.12 Conclusion

My research hypothesis stated that, firstly, calcitriol is not required for bone and mineral homeostasis. Secondly, we hypothesized that the upregulation of placental function in the *Vdr* null fetuses may be due to calcitriol having physiological effects to reduce placental calcium transfer, such that when calcitriol has no receptor to signal through, calcium transport increases due to loss of calcitriol's normal effects as a "brake" on the forward flow. Or alternatively, we hypothesized that since *Vdr* null fetuses have higher than normal concentrations of calcitriol, it is conceivable that such high levels of calcitriol act through non-classical receptors to upregulate placental calcium transport despite the absence of VDR. My studies of *Cyp27b1* null fetuses agree with our first hypothesis that calcitriol is not required for bone and mineral homeostasis. My studies also agree with the

alternate version of the second hypothesis, that calcitriol can act through non –classical receptors to upregulate placental calcium transport despite absence of VDR.

To conclude, fetal-sourced calcitriol is not required to regulate fetal bone and mineral homeostasis. However, when the current findings are contrasted with those of *Vdr* null fetuses, which have abnormally high concentrations of circulating calcitriol, it is apparent that calcitriol may be able to act on an alternate receptor, that has not yet been identified, in order to explain the upregulation of placental function that occurs in *Vdr* null fetuses and with pharmacological treatment with calcitriol, as opposed to the lack of upregulation in placental calcium transport and gene expression that occurs in fetuses that lack vitamin D or calcitriol. Loss of maternal calcitriol caused modest effects on the fetal phenotype that were independent of fetal calcitriol levels, but, these effects were relatively masked by the ability of the placenta and fetus to maintain bone and mineral homeostasis without fetal calcitriol. Whether these modest effects of loss of maternal calcitriol on fetal bone and mineral homeostasis have any long-term effects on neonatal, child, or adult mineral and bone metabolism, are beyond the scope of this MSc thesis and remain the subject of future investigations.

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