Role of calcitriol in regulating fetal mineral homeostasis and bone development

by

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ABSTRACT

Calcitriol has a critical role in regulating mineral and bone metabolism in children and adults. However, its role in fetal mineral and bone metabolism is not yet well understood. Hence, we investigated the role of calcitriol during fetal development using a knockout mouse model involving ablation of the *Cyp27b1* gene encoding 1α-hydroxylase that converts 25-hydroxyvitamin D_3 to 1,25-dihydroxyvitamin D_3 (calcitriol). Specifically, this study compared *Cyp27b1* null fetuses to wild type (WT) fetuses from heterozygous (*Cyp27b1*$^{+/-}$) mothers. Our findings showed that *Cyp27b1* null fetuses had normal calcium and phosphorus levels in serum and amniotic fluid, normal placental mineral transport, as well as normal skeletal ash weight, mineral content and bone morphology. The quantitative RT-PCR confirmed the loss of *Cyp27b1* expression in null fetal kidneys, but there were no changes in fetal renal or placental expression of calciotropic and phosphotropic genes. Although the calcitriol level in *Cyp27b1* null fetuses was significantly lower than the WT siblings, it was not undetectable, likely due to placental transfer of maternal calcitriol to the fetuses. To investigate this possibility, a preliminary study was conducted to assess the fetal mineral and bone metablism of *Cyp27b1* null fetuses born from *Cyp27b1* null mothers. Pilot data showed that these fetuses had normal serum calcium and skeletal ash weight and morphology. In summary, our findings herein suggest that calcitriol may not play a role in fetal mineral and bone metabolism. However, further studies involving *Cyp27b1* null fetuses from null mothers are required to conclusively support this conclusion.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

% of +/- Mean	Percent of heterozygous mean
+/+ or WT	Wild type
+/- or HET	Heterozygous
-/- or Null	Homozygous
$1,25(OH)_2D_3$	1,25- Dihydroxyvitamin D_3 (Calcitriol)
1α(OH)ase	1-alpha-hydroxylase
7-DHC	7-Dehydrocholesterol
24OH	24-Hydroxylase
$24,25(OH)_2D_3$	24,25-dihydroxyvitamin D_3
$25(OH)D_3$	25-Hydroxyvitamin D_3
ADHR	Autosomal dominant hypophosphatemic rickets
ALP	Alkaline Phosphatase
ANOVA	Analysis of Variance
BMPs	Bone morphogenetic proteins
BRC	Bone Remodeling Compartment
bp	Base Pair
Ca	Calcium
Ca^{2+}ATPase	Calcium Adenosinetriphosphatase
CaBP	Calbindins
CaBP-9k	Calbindin-D9K (i.e. S100g)
CaSR	Calcium-Sensing Receptor
cDNA	Complementary DNA

cFMS	colony stimulating factor-2 receptor
cm	Centimeter
C_T	Threshold Cycle
C-terminal	Carboxy-terninal (i.e. COOH-terminal)
CCAC	Canadian Council on Animal Care
Cr	Chromium
CYP	Cytochrome P450
CYP27A1	CYP family 27 subfamily A member 1
CYP2R1	CYP family 2 subfamily R member 1
Cyp27b1	Cytochrome P450 Family 27 Subfamily B Member1
CYP2D25	CYP family 2 subfamily D member 25
DNA	Deoxyribonucleic Acid
dATP	Deoxyadenosine Triphosphate
dCTP	Deoxycytidine Triphosphate
dGTP	Deoxyguanosine Triphosphate
dNTPs	Deoxynucleotide Triphosphates
dTTP	Deoxythymidine Triphosphate
ECF	Extracellular fluid
ED	Embryonic Day
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
EtOH	Ethanol
FGF23	Fibroblast growth factor 23

HHRH	Hereditary hypophosphatemic rickets with hypercalciuria
HVDRR	Hereditary vitamin D-resistant rickets
IACC	Institutional Animal Care Committee
IFN	Interferon
IL	Interleukin
L	Liter
M	Molar
MBq	Millibecquerel
MSCs	Mesenchymal Stem Cells
µCi	Microcurie
µg	Microgram
µl	Microliter
ml	Milliliter
mM	Millimolar
mmol	Millimoles
mRNA	Messenger Ribonucleic Acid
n	Number of Observations
NaCl	Sodium Chloride
NaPi2a/2c	Sodium-phosphate cotransporters
NS	Not-Significant
OH	Hydroxide
p	Probability
PCR	Polymerase Chain Reaction

Pdia3	protein disulfide isomerase family A, member 3
Pg	Picogram
Pi	Phosphate
PMCA1	plasma membrane calcium ATPase
PTH	Parathyroid Hormone
PTHR1	Parathyroid hormone-related protein receptor type 1
PTHrP	Parathyroid hormone-related protein
qPCR	Real-Time Quantitative Reverse Transcriptase-PCR
RANKL	Receptor Activator of NF-κB Ligand Ligand
RNA	Ribonucleic Acid
RXR	Retinoid X receptors
SD	Standard Deviation
SEM	Standard Error of the Mean
TGF- ß	Transforming growth factor ß
TNF	Tumor necrosis factor
TRPV6	Transient Receptor Potential Cation Channel, Subfamily V, Member 6
VDR	Vitamin D Receptor
XLH	X-linked hypophosphatemic

1 INTRODUCTION

1.1 Preamble

This study was done to investigate the role of 1,25-dihydroxyvitamin D_3 (calcitriol) in regulating mineral homeostasis and bone metabolism in the fetus. Previous studies in our lab and other studies suggest that unlike its critical role in infants, children and adults, calcitriol might not be required for fetal bone and mineral metabolism. To investigate this hypothesis, we studied mice unable to make calcitriol due to ablation of the cytochrome P450 family 27 subfamily B member 1 (*Cyp27b1*) gene, which encodes 1α-hydroxylase, the enzyme required to synthesize calcitriol. To understand the aim of this project, this introduction will provide background information on the specific role of Vitamin D, and its associated hormones, in the regulation of bone and mineral metabolism in adults and fetuses.

Throughout this thesis the standard nomenclature for naming genes and proteins will be followed. Human genes and proteins will be in all capital letters while animal genes and proteins will be in lower case, with the initial capital letter only. Both human and animal genes will be italicized, while proteins are in standard font.

1.2 Vitamin D: Metabolism and Mechanism of Action

Vitamin D_3 is derived from the diet or is synthesized in the skin by ultraviolet irradiation of 7-dehydrocholestrol (1). Vitamin D_3 has no biological activity on its own, and the active form of vitamin D_3, 1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$; also known as calcitriol), is produced following hydroxylation of the 1^{st} and 25^{th} carbon atoms of vitamin D_3 (2). The hydroxylation of the 1^{st} and 25^{th} carbons of vitamin D_3 occurs in the liver and kidneys, respectively (Figure 1).

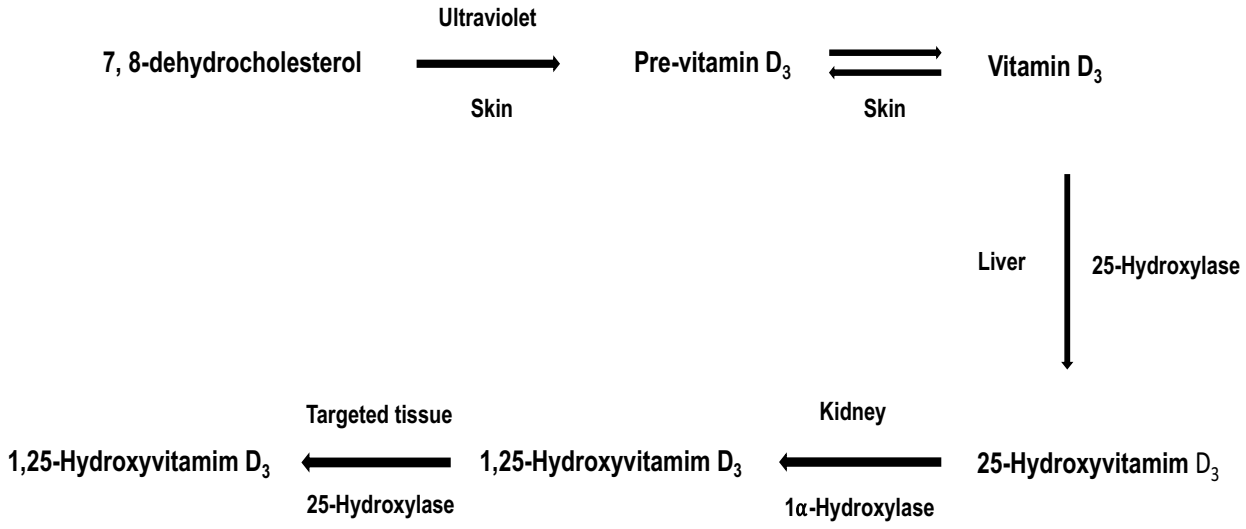

Figure 1. Vitamin D metabolism pathway.

Vitamin D_3 is converted to its active metabolite, 1, 25-dihydroxyvitamin D_3 (calcitriol), following hydroxylation in the liver and kidneys. Adapted with permission from (3) Appendix A.

In the liver, C-25 of vitamin D_3 is hydroxylated to produce 25-hydroxyvitamin D_3 ($25(OH)D_3$) by the actions of cytochrome P450 (CYP) enzymes such as CYP family 2 subfamily R member 1 (CYP2R1), CYP family 27 subfamily A member 1 (CYP27A1), and CYP family 2 subfamily D member 25 (CYP2D25; (1,2). However, it has been suggested that CYP2R1 is the key enzyme, as patients with homozygous mutations of *CYP2R1* are known to have low levels of $25(OH)D_3$ and classical symptoms of vitamin D_3 deficiency (4).

Subsequent to the blood transport of $25(OH)D_3$ by the vitamin D binding protein, the second hydroxylation at C-1 position occurs in the kidney, where $25(OH)D_3$ is converted into calcitriol by the $25(OH)D_3$ 1α-hydroxylase (also known as CYP27B1) enzyme (5). CYP27B1, which is predominantly present in the proximal renal tubule (1), is considered the principle enzyme in the formation of calcitriol. The physiological importance of calcitriol is evident from human disorders such as vitamin D-dependent rickets (VDDR) type I and type II. In VDDR type I, where calcitriol is absent, non-synonymous homozygous mutations of *CYP27B1* are known to cause the phenotype, also referred to as pseudovitamin D deficiency rickets. In VDDR type II, inactivating mutations of the vitamin D receptor make it non-functional and lead to the phenotype, also known as hereditary vitamin D-resistant rickets. While both conditions cause rickets, seizures, hypocalcemia, secondary hyperparathyroidism, they differ in the genetic cause and the fact that only VDDR type II causes total scalp and body alopecia in some but not all cases (6).

The activity of CYP27B1 and synthesis of calcitriol in the kidney is tightly controlled by actions of parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23), and calcium and phosphate

concentrations (2,6). Specifically, low calcium increases PTH which stimulates the transcription of CYP27B1 and thereby the production of calcitriol, whereas FGF23 and calcitriol inhibit CYP27B1 (2). The existence of CYP27B1 at extra-renal sites has also been widely documented (7). The extra-renal production of calcitriol in tissues, such as keratinocytes, macrophages, and osteoblasts, is controlled by actions of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukin (IL)-1β (1,6). The expression of *CYP27B1* has also been documented in fetal trophoblasts and maternal decidua (8).

The 24-hydroxylase mitochondrial enzyme, CYP family 24 subfamily A member 1 (CYP24A1) is also involved in vitamin D metabolism via hydroxylation of both $25(OH)D_3$ and $1,25(OH)_2D_3$ (1). CYP24A1 limits the amount of $1,25(OH)_2D_3$ in target tissues both by accelerating the catabolism of $1,25(OH)_2D_3$ to $1,24,25(OH)_3D_3$, resulting in calcitroic acid, and by producing $24,25(OH)_2D_3$ and $23,25(OH)_2D_3$, thereby reducing the pool of $25(OH)D_3$ available for 1-hydroxylation (2,5,9). In humans, *CYP24A1* mutations have been reported to have a causal role in patients with idiopathic infantile hypercalcemia, providing further evidence of the role of CYP24A1 in $1,25(OH)_2D_3$ catabolism (9,10).

The physiological effects of calcitriol are primarily known to be mediated by actions on its classical receptor, the vitamin D receptor (VDR) (1). The VDR belongs to the steroid receptor family that encompasses retinoic acid, thyroid hormones, sex hormones, and adrenal steroid receptors (11). The stereospecific binding of calcitriol to the VDR results in heterodimerization with the retinoid X receptors (RXR) (12). Formation of the calcitriol-VDR-RXR complex results

in the activation of transcription factors or co-activators, such as TATA binding protein-associated factors, p160 coactivators and steroid receptor activators (12).

Vitamin D receptors are widely expressed throughout the body in classical target tissues such as osteoblasts, enterocytes, distal renal tubule cells, the parathyroid gland, keratinocytes, promyelocytes, lymphocytes, the pituitary gland and ovarian cells, to name only a few (5). Hence, it has been suggested that calcitriol may have extraskeletal physiological roles; however, this remains to be elucidated. The following sections of my thesis will focus on the role of calcitriol-VDR signalling in the regulation of mineral homeostasis and bone development in adults and fetuses.

1.3 Mineral Homeostasis

The classical endocrine actions of calcitriol, in concert with PTH and FGF23, are predominantly responsible for the regulation of calcium and phosphorus homeostasis (Figure 2; (1). Calcium, phosphate, and magnesium are multivalent ions that are vital for many biological and cellular functions (13). The imbalance in the maintenance of mineral homeostasis can be manifested by numerous clinical symptoms [reviewed in detail elsewhere (14)]. Calcium is the most abundant mineral in the body, and together with phosphorous, they comprise over 90% of the minerals in bone (15). The homeostatic balance of calcium and phosphorus are tightly regulated and maintained by complex interactions of hormonal and non-hormonal factors, dietary intake and functional abilities of the renal, gastrointestinal and skeletal systems (14).

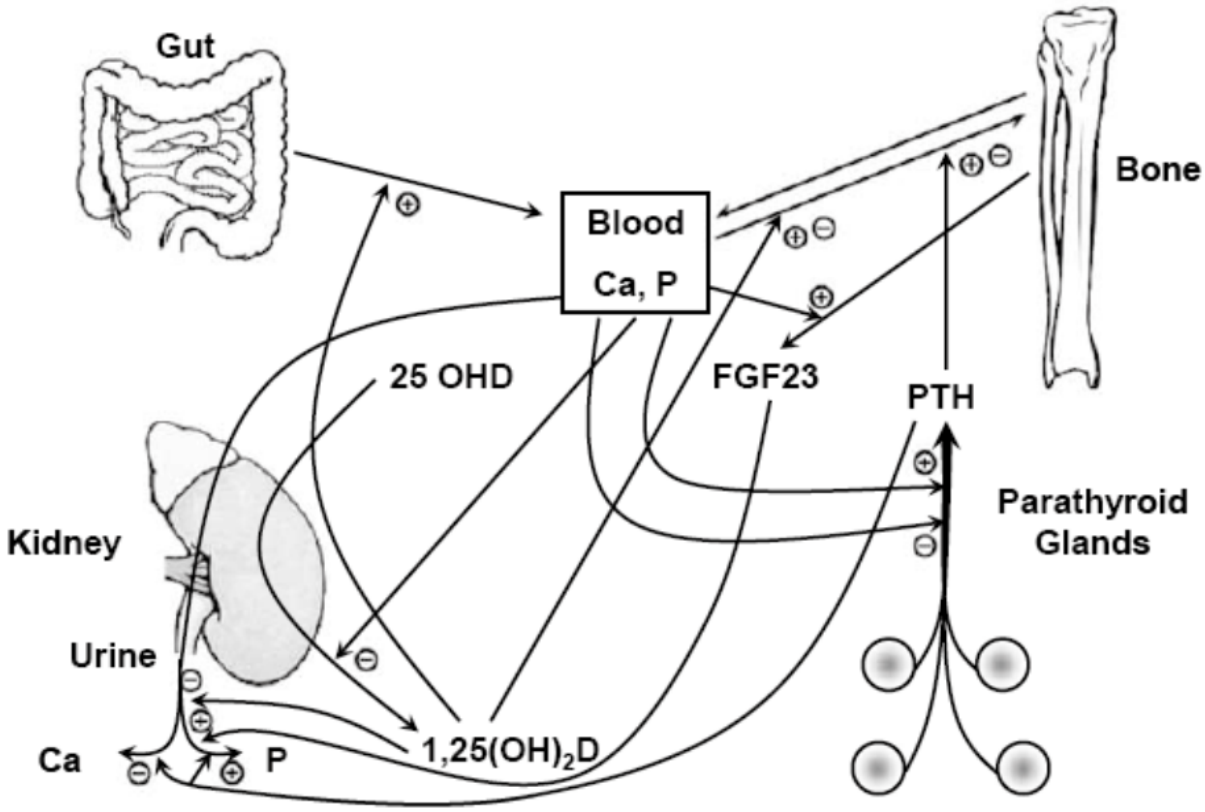

Figure 2. Role of calcitriol in mineral homeostasis in adults.

Mineral homeostasis is regulated by calcitriol and PTH actions in the intestines, bone and kidneys. (+) and (-) signs mean stimulate and inhibit, respectively. An increase in calcium suppresses PTH secretion while an increase in phosphorus stimulates it. Calcitriol increases intestinal calcium absorption and renal calcium reabsorption. PTH increases phosphorus excretion by kidneys and promotes renal calcium reabsorption. Calcitriol interacts with PTH and FGF23 to maintain Ca and P. An increase in serum Ca inhibits PTH while a decrease in serum Ca or a rise in serum P stimulates it. Reproduced with permission (12) Appendix B.

1.3.1 Role of Calcitriol in Calcium and Phosphorus Homeostasis in Adults and Fetuses

1.3.1.1 Calcitriol and Calcium Homeostasis in Adults

Calcium is an essential element in bone mineralization and plays a critical role in many biological functions including cell signaling and cellular membrane integrity, muscle contractions, nerve function, glycogen metabolism, and blood clotting (13,14,16). In adults, the human body contains about 1000 g of calcium, ~99% of which is located in the mineral phase of bone as hydroxyapatite crystals [$Ca_{10}(PO_4)_6(OH)_2$ (17)]. The remaining 1% of the total calcium in the body is located in the blood, extracellular fluids and soft tissues (16). Calcium in the blood is normally transported in a free/ionized state (45%), bound to phosphate/citrate (10%) and bound to plasma proteins (45%) (13).

In adults, serum calcium (Ca^{2+}) concentrations are tightly regulated by actions of the Ca^{2+}-sensing receptor (CaSR), PTH and calcitriol (18), with each of these hormones networked by a modulatory feedback loop mechanism. Serum calcium levels are mainly maintained by the CaSR, which belongs to the family C of G-protein-coupled receptors located in the parathyroid gland. The CaSR directly regulates calcium reabsorption in the kidneys. Low serum Ca^{2+} concentrations lead to less activation of CaSR (18). The resultant increase in PTH hormone further stimulates the production of calcitriol, which regulates intestinal calcium absorption and kidney calcium reabsorption (19). Calcitriol also suppresses PTH by increasing serum Ca^{2+} concentration, which acts on the CaSR in the parathyroid gland (16). PTH can also reduce renal calcitriol degradation by reducing the half-life of *CYP24A1* mRNA (20). In the enterocytes of the intestinal tract, calcitriol increases the expression of the transient receptor potential cation channel, subfamily V, member 6 (TRPV6), and plasma membrane Ca^{2+} ATPase (PMCA1),

thereby facilitating transcellular calcium uptake (1). At the renal distal tubules, calcitriol increases the intracellular expression of calbindin-D_{28K}, expression of TRPV member 5 (TRPV5) at the apical membrane, and expression of the ATP-dependent calcium transporter at the basolateral membrane, resulting in enhanced PTH-dependent calcium-reabsorption from the glomerular filtrate (21). Calcitriol is also known to upregulate klotho, a co-factor in the FGF23 signalling pathway, and on the contrary, the loss of klotho results in the induction of Cyp27b1 and calcitriol (22). The latter notion is supported by *in vivo* findings showing hyperphosphatemia and increased calcitriol in *Klotho*-deficient mice and *Fgf23*-deficient mice (22). The normal serum calcium levels in *Klotho*- and *Fgf23*-deficient mice validate the notion that FGF23 does not play a role in calcium homeostasis (19). Although, a study by Andrukhova O, et al, reported that FGF23 enhances calcium reabsorption in kidneys through the TRPV5 channel (23).

It is well established that the physiological actions of calcitriol are mediated by its actions on the VDR, encoded by the *VDR* gene (5). Studies in humans have shown reduced calcium absorption in patients with severe vitamin D deficiency (24,25), specifically when secondary hyperparathyroidism is unable to maintain serum calcitriol levels. Indeed, mutations of *VDR* (26,27) and *CYP27B1* (28,29) in humans have been known to cause severe hypocalcemia as well as secondary hyperparathyroidism and hypophosphatemia. To date, several rodent models of vitamin D deficiency that mimic phenotypic characteristics observed in humans have been created by several groups involving the ablation of *Vdr* and *Cyp27b1* (30).

The robust role of calcitriol in calcium homeostasis is supported by findings from vitamin D-deficient animals, and global *Vdr* and *Cyp27b1* null mice showing reduced calcium absorption,

resulting in severe hypocalcemia as well as secondary hyperparathyroidism and hypophosphatemia (30-32). In the intestinal-specific deletion of *Vdr* null mice, intestinal calcium absorption is decreased. However, these mice show normal blood calcium, which is different from the systemic *Vdr* null mice having low levels of serum calcium (33). The normal values of serum calcium in the intestinal-specific deletion of *Vdr* null mice are the result of bone resorption and impaired mineralization (with high calcitriol levels affecting the mineralization process by osteoblasts), whereas the osteomalacia in the systemic *Vdr* nulls is a result of the hypocalcemia. However, the fact that the abnormal calcium homeostasis observed in *Vdr* (34) and *Cyp27b1* null mice (35-39), and in humans (29,40,41) can be prevented by dietary calcium supplements suggests that the main role of calcitriol with respect to the skeleton is indirect and is simply to stimulate calcium and phosphorus absorption in the intestine.

The homozygous ablation of *Vdr* and *Cyp27b1* has also been shown to reduce the expression of calcium transport proteins, such as Trpv6 and Pmca1, thereby reducing Ca^{2+} absorption in the intestine of adult mice (42-45). Likewise, impaired renal Ca^{2+} reabsorption due to a reduction in the expression of calbindinD-$_{9k}$ (CaBP-9k; S100g) and Trpv5 has been documented in adult *Vdr* and *Cyp27b1* null mice (30). However, the unaffected calcium absorption in *Cabp-9k*, *Trpv5*, *Trpv6* and *Pmca1b*-deficient mice suggests redundancy or the involvement of mediators other than calcitriol in intestinal calcium absorption (30).

The actions of calcitriol-VDR signalling in bone largely depend upon calcium levels. In response to hypocalcemia, the actions of calcitriol promote osteoclastic bone resorption by binding to receptors in the preosteoblastic stromal cell and stimulating the Receptor Activator of Nuclear

Factor-κB (RANK) /RANK ligand (RANKL) system and facilitate calcium turnover to the extracellular fluid (13). Calcitriol induces the expression of RANKL and suppresses the expression of osteoprotegerin, which inhibits the effects of RANKL (46). The calcitriol/VDR system may also inhibit the mineralization of bone indirectly by releasing a phosphaturic factor FGF23 from osteocytes (47). On the contrary, the calcitriol/VDR system increases bone formation in the presence of normocalcemia. Thus, the VDR specifically overexpressed in mature osteoblasts enhances bone formation and decreases bone resorption in vivo (48). Furthermore, the overexpression of VDR in osteoblasts and osteocytes has been reported to prevent bone loss due to vitamin D-deficiency (49). In somewhat discordant findings, the specific deletion of *Vdr* in osteoblasts has been shown to be associated with reduced bone resorption but to cause a 20% increase in bone mass (50). This reflects the cross-talk between osteoblasts and osteoclasts, and that a reduction in bone resorption can be expected to result in a net increase in bone mass. Overall, physiologic levels of calcitriol in mature osteoblasts are required to promote bone formation, with effects on osteoblast proliferation and enhanced mineralization, whereas higher calcitriol levels favor bone resorption (51).

Taken together, calcitriol-mediated VDR signalling is required for the regulation of calcium homeostasis in adults by promoting intestinal absorption and bone turnover of calcium. Thus, calcium homeostasis in the adult human body is maintained by orchestrated actions of calcitriol, CaSR and PTH present in renal, gastrointestinal and skeletal tissues.

1.3.1.2 Calcitriol and Phosphorus Homeostasis in Adults

Phosphorus is an important element in bone mineralization and plays a vital role in multiple biological processes (52). Phosphorus is mostly stored in bone (80-90%) where it binds to calcium to form hydroxyapatite crystals (53). The remaining (10-20%) is found in extracellular fluid (ECF) in the form of organic phospholipids and inorganic phosphate (Pi) (54). The normal blood levels of phosphorus in adults range between 2.5-4.5 mg/dl, with higher phosphorus concentrations usually observed in men compared to women.

Phosphorus homeostasis in adults is maintained by actions of PTH, FGF23 and calcitriol that act on the intestines, kidneys and bone tissues (55,56). PTH increases serum phosphate either directly, by stimulating bone turnover and phosphate release, or indirectly, by facilitating intestinal phosphate absorption via increased calcitriol production (57). On the other hand, the activation of renal parathyroid hormone receptor type 1 (PTHR1) by PTH and the subsequent activation of the cyclic adenosine monophosphate (cAMP) and phospholipase C leads to internalization of the sodium–phosphate cotransporters NaPi2a and NaPi2c, thereby reducing renal phosphate reabsorption (1,14,57). In bone cells, intermittent PTHR1 signalling stimulates bone formation, whereas sustained PTH/PTHR1 signalling stimulates RANKL production, which in turn increases the osteoclast number, bone resorption, and, subsequently, phosphate release (16,57).

The pivotal role of FGF23 in the regulation of phosphate homeostasis was revealed when studying genetic disorders (discussed in detail below) where serum calcitriol was normal but the values were inappropriately low given that serum phosphorus was low. This led to the

conclusion that something was promoting renal phosphate wasting and also suppressing calcitriol synthesis or increasing its degradation. A loss of function mutation of *FGF23* in humans (58) was identified. This was also observed in studies of mice lacking *Fgf23* (59) or the co-receptor *Klotho* (60). FGF23 is predominantly produced by osteocytes and osteoblasts and regulated by serum phosphate levels and dietary phosphate intake. In the kidneys, FGF23 reduces the expression of NaPi2a and NaPi2c to promote phosphorous excretion. It also reduces the expression of CYP27B1 and increases the activity of CYP24A1 in the proximal tubules, thereby reducing the production and increasing the catabolism of calcitriol, respectively, in the kidneys (61). In this way it acts to indirectly lower phosphate absorption in the intestine (59).

The overexpression of *Fgf23* and injection of FGF23 into mice causes hypophosphatemia and reduces calcitriol levels, and, inversely, mice lacking *Fgf23* have hyperphosphatemia and high calcitriol (30). However, an increase in FGF23 levels following the administration of calcitriol and subsequent to the normalization of serum calcium and phosphate levels by dietary means in *Vdr* null mice suggests the regulation of FGF23 by both calcitriol/VDR-dependent and –independent signalling pathways (2,12,30).

The discovery of various genetic causes of human hypo- and hyper-phosphatemic disorders, and the subsequent development of rodent models mimicking these conditions, have facilitated our understanding underpinning phosphate homeostatic regulation. Hypophosphatemia causes accumulation of osteoid in children, resulting in severe mineralization defects in the zone of provisional calcification in the growth plates (rickets; (54). In adults, hypophosphatemia leads to accumulation of osteoid in cancellous and cortical bone (osteomalacia; (54). Human

physiological states that affect phosphate homeostasis include autosomal dominant hypophosphatemic rickets (ADHR), X-linked hypophosphatemic (XLH) rickets/osteomalacia and hereditary hypophosphatemic rickets with hypercalciuria (HHRH), to name only a few (54). XLH rickets/osteomalacia is an archetypal phosphate-wasting disorder characterized by low serum calcitriol and normal PTH levels, in the context of low serum phosphorous, along with progressive skeletal abnormalities and growth retardation (62).

The phenotypic and biochemical manifestations of patients with ADHR are similar to those of patients with XLH. However, patients with ADHR display variable incomplete penetrance of a renal tubular abnormality as well as the delayed onset of penetrance (63). HHRH is a rare genetic disease characterized by hypophosphatemia due to increased renal phosphate clearance and normocalcemia, and increased serum calcitriol (64). The elevated calcitriol levels enhance intestinal calcium absorption, increase renal calcium load and inhibit PTH, resulting in hypercalciuria (64).

Hyperphosphatemia on the other hand is characterized by high calcitriol and increased intestinal phosphate absorption. Genetic disorders such as tumoral calcinosis, characterized by loss of *FGF23,* or periarticular cystic tumorous calcifications, from loss of *KLOTHO,* lead to elevated serum calcitriol and normal serum PTH levels, which causes hyperphosphatemia (65). Furthermore, human clinical conditions such as renal insufficiency, malignancy, drug abuse, or hypoparathyroidism can also lead to hyperphosphatemia (16). Specifically, several clinical hypophosphatemic and hyperphosphatemic conditions are known to be associated with elevated FGF23 levels. However, the elevated FGF23 levels observed in patients with hyperphosphatemic

conditions are believed to be in response to the hyperphosphatemia, rather than being the cause of this condition (66). And while the high FGF23 levels counteract the hyperphosphatemia by reducing the levels of phosphate it doesn't lower phosphate to normal physiological levels.

Unlike the major role of PTH and FGF23 in phosphate homeostasis, only ~30% of intestinal phosphorus absorption is regulated by the actions of calcitriol (56). Calcitriol is known to regulate intestinal phosphorus absorption by increasing the expression of *NAPI2B* (67,68). Calcitriol may also regulate phosphate homeostasis by increasing *KLOTHO* expression in the distal renal tubule (69). The role of the calcitriol-VDR signaling pathway in phosphorus homeostasis is substantiated by the presence of hypophosphatemia in *Vdr* and *Cyp27b1* null mice (31,32). However, it is possible that this interpretation may be confounded by the presence of secondary hyperparathyroidism in *Vdr* and *Cyp27b1* null mice, which may have contributed to the hypophosphatemia by suppressing NaPi2a/c transporters (70). The actions of calcitriol have been attributed to the maintenance of phosphate absorption in the intestine. This is evidenced by the complete rescue of the osteomalacia/rickets phenotype in *Vdr* and *Cyp27b1* null mice (34,36,39) given a diet high in calcium and phosphorus. There is also evidence of an indirect role of calcitriol-VDR signaling in the maintenance of phosphorus concentrations in bone. This notion is further supported by the ability of calcitriol to stimulate the synthesis of FGF-23 by osteoblasts and osteoclasts, which acts on the kidney to promote phosphate excretion by decreasing the renal expression of sodium-phosphate cotransporters (NaPi2a/c; (71). It is also possible for calcitriol to exert its anti-rachitic effects in bone indirectly by stimulating intestinal calcium and/or phosphorous absorption (34).

1.3.1.3 Calcitriol and Mineral Homeostasis in Fetuses

It has been established that fetal blood calcium and phosphorus homeostasis are regulated differently from adults. However, little is understood about this mechanism due to the obvious limitations in studying human fetuses. Unlike adults, the calcium and phosphorus homeostatic mechanism in fetuses does not appear to involve many of the hormones that have a crucial role in adults.

A consistent finding across various mammalian fetuses, including humans, rhesus monkeys, sheep, cattle, rodents and pigs, is that the serum calcium concentration is maintained at 0.30 - 0.50 mmol/L higher than in maternal serum (5,72,73). The presence of high fetal blood calcium levels suggests its importance in fetal physiology. However, studies involving transgenic mice with ablation of various calciotropic genes, such as *Pthrp*, *Pthr1*, Homeobox A3 (*Hoxa3)*, *Trpv6* and *Hoxa3/Pthrp*, have demonstrated that the presence of fetal hypocalcemia, due to ablation of these genes, does not affect fetal viability (74-76). The reduced skeletal calcium content of homozygous fetuses (e.g., $Pthrp^{-/-}$, $Pthr1^{-/-}$ and $Hoxa3^{-/-}$) with hypocalcemia underlines the importance of high fetal serum calcium to promote normal skeleton mineralization *in utero* (74-76).

The high level of blood calcium in fetuses is always maintained regardless of the presence of maternal hypocalcemia. This notion is supported by rodent studies showing high fetal calcium despite maternal hypocalcemia (77), maternal vitamin D deficiency (78,79) and maternal thyroparathyroidectomy(80,81). Similarly, normal fetal hypercalcemia is robustly maintained despite maternal vitamin D deficiency or hypoparathyroidism in humans (82). In contrast, there

is evidence that maternal hypercalcemia raises fetal blood calcium levels, both in the acute and chronic manner in rodents (73).

Fetal serum phosphorus levels are also maintained at ~0.50 mmol/L higher than the mother in humans, rats, mice, sheep and pigs (83). Fetal phosphorus levels remain unaffected despite maternal hypophosphatemia in rodents (84,85). However, hyperphosphatemia in pregnant rats and sheep has been shown to cause fetal hyperphosphatemia (73,86,87).

Calcitriol in fetuses typically circulates <50% of the maternal value in fetal humans, rodents and pigs (88-90). The contribution of the fetal kidneys is significant because fetal nephrectomy reduced the fetal calcitriol levels in sheep and rats (91,92). In humans, umbilical artery levels of calcitriol are higher than umbilical venous levels, indicating the contribution of the human fetal kidneys (93). Maternal nephrectomy in rats also confirmed the independent synthesis of calcitriol in the fetal-placental unit (94). In agreement with this notion, studies have shown the expression of *CYP27B1* and *CYP24A1* by the placenta and fetal kidneys in both humans and rodents (73,95).

Previous studies suggest that calcitriol does not cross the placenta. This evidence includes no significant increase in the serum calcitriol of *Cyp27b1* null Hanover pigs (88) from *Cyp27b1*$^{+/-}$ dams and the inability of radiolabeled calcitriol to cross rat placentas (90). In contrast, calcitriol has been shown to cross sheep placentas (96) and perfused human placentas (97), indicating possible between-species differences in the ability of calcitriol to cross the placenta.

To date, a number of studies have established that neither vitamin D nor calcitriol are required for the maintenance of fetal calcium and phosphorus homeostasis (73,83). However, the pivotal role of calcitriol in regulating calcium and phosphorus homeostasis in neonates, children and adults is well-established (73,83). Fetal blood calcium and phosphorus levels remain normal despite severe vitamin D deficiency in rats and following homozygous ablation of *Vdr* and *Cyp27b1* in mice and pigs (31,98,99). Furthermore, fetal nephrectomy in rats has also been shown to not affect blood calcium or phosphorus levels (100). The cord blood of human fetuses from severely vitamin D deficient mothers has been demonstrated to have normal calcium and phosphorus levels (73,83,101,102). In a double-blind clinical and meta-analysis, the administration of vitamin D to pregnant mothers resulted in a significant increase in fetal levels of calcitriol in vitamin D-treated mothers compared to placebo-treated mothers. However, there was no difference in cord blood calcium or phosphorus between groups (103,104).

The placenta is the main source of minerals in the fetus. Consequently, the transfer of minerals across placenta also contributes to fetal mineral homeostasis (105). *Vdrs* are expressed in the placenta of sheep (106), humans (107,108), rats (109) and mice (110), and in the trophoblasts and yolk sac of mice (73,110). Pharmacological doses of calcitriol have been shown to increase calcium transfer in an in-situ placental perfusion models in rats (111), guinea pigs (112), and sheep (111-113). Prior nephrectomy of fetal sheep reduced calcium placental transfer, with this effect restored following the administration of calcitriol (114). Collectively these findings suggest a role for calcitriol-VDR signalling in the regulation of placental mineral transport. However, calcitriol may not be required for placental calcium transport as calcium delivery was normal in severely vitamin D-deficient fetal rats (115). Both severely vitamin D-deficient rats

and *Vdr* null mice display normal placental expression of S100g and Pmca1, vital regulators of placental transfer of calcium (17,31,115,116). However, *Vdr* null fetuses have increased placental calcium transport, high levels of calcitriol, and increased placental expression of *Pthrp* and *Trpv6* (31,117) . Thus, it plausible that the high level of calcitriol is acting through receptors other than the classical VDR. If so, this phenomenon could explain why placental calcium transport, and the expression of *Pthrp* and *Trpv6*, were increased in *Vdr* nulls. Alternatively, it is possible that calcitriol normally acts to inhibit placental calcium transport, such that the loss of its receptor causes an increase in the rate of transport and its associated gene expression.

1.4 Skeletal Development

The skeletal system has several functions including structural support, the protection of internal soft tissues, working with the muscles to allow the body to be flexible for movement, producing blood cells from bone marrow and maintaining serum mineral levels through the bone formation and resorption process (118). Bone is a highly specialized connective tissue which forms the skeleton of all vertebrates (119). Bone tissue is essentially composed of three major components: collagen fibrils, mineral particles and water (119). The mineral particles in bone are composed of calcium phosphate, which exists in the form of carbonated apatite in vertebrates (119).

Bone is divided into two main types–cortical and trabecular. The first type, cortical bone (also known as compact bone) is made of numerous overlapping cylindrical units (osteons; (120). It is mainly found in the shafts of long bones and represents around 80% of bone mass in the human body. Compact bone provides the strength and structure necessary to protect the inner organs. The second type, cancellous or trabecular bone, has an open honeycomb structure and accounts

for roughly 20% of the total bone mass (120). It is found at the end of the long bones of the appendicular skeleton and in the vertebral bodies of the axial skeleton. It is composed of a meshwork of trabeculae, thereby reducing the skeletal weight without compromising strength. It consists of trabeculae surrounding many red marrow-filled spaces and has metabolic activities (121).

Bone development in the human fetus requires approximately 30 g of calcium during gestation, which is actively transported across the placenta (122). The skeleton starts to form at 20 weeks of pregnancy, with approximately 60 mg of calcium accreted per day by 25 weeks (122). The calcium deposited in the fetal skeleton increases to more than 300 mg per day between the 35^{th}-38^{th} weeks and gradually decreases in the 39th and 40th weeks (122). Similarly, term fetal mice have ~1.5-2.0 mg of calcium, ~70-80% of which is accreted during the last four days of the 19-day gestation period (122).

Patterning of the early embryonic skeleton is dependent upon a multitude of signaling pathways, including Hox genes, Wnts, Hedgehogs, bone morphogenetic proteins, fibroblast growth factors and Notch/Delta, to name only a few (123). Primary ossification centers form in the vertebrae and long bones between the 8th and 12th week of embryo development in humans. However, the bulk of mineralization does not occur until the third trimester (124). In mice, primary ossification centers are present in most endochondral bones at term, whereas secondary ossification centers only develop after birth (73).

There are two important processes that occur during fetal skeletal development and the

formation of bone tissues (osteogenesis): 1) intramembranous (mesenchymal) ossification and 2) endochondral ossification. Most of the skeleton forms through the multistep process of endochondral bone formation, where mesenchymal progenitors differentiate into chondroblasts and chondrocytes. In contrast, in few places, such as the flat bones of the skull, bones form through the intramembranous pathway, where the mesenchymal cells differentiate directly into osteoblasts which then form bone (119).

The process of endochondral ossification is triggered by the differentiation of proliferating chondrocytes and is followed by the invasion of osteoblast progenitors, osteoclasts, endothelial cells and hematopoietic cells from the perichondrium into the hypertrophic cartilage (119,123). Osteoblast progenitors in the perichondrium differentiate into osteoblasts, and the primary ossification center expands and secondary ossification centers form, resulting in formation of the epiphyseal growth plate (125). In the growth plates, the chondrocytes are organized into structural and functional zones (Figure 1) and undergo clonal expansion. With age, the growth plates get thinner and are eventually replaced by bone at various times after puberty in humans.

Bone tissues are formed by three primary cell types: osteoblasts, osteocytes and osteoclasts. These cells are responsible for bone formation and degradation. Each cell type has a unique function and is found in different locations within bone. The osteoblasts, located along the bone surface, represent 4-6% of total bone cells and are mostly known for their bone formation function (126,127). Osteoblasts are derived from mesenchymal stem cells (MSCs) by the action of a multitude of cytokines. Osteoclasts make up the smallest percentage (about 1-2%) of bone cells in the human skeleton. These multinucleated cells are responsible for resorption and degradation of the mineralized bone matrix (128). Osteoclasts are derived from mononuclear

cells of the hematopoietic stem cell lineage (monocyte/macrophage family) (129). Osteocytes represent approximately 90–95% of total bone cells with a lifespan of up to 25 years (130). Osteocytes originate from the terminal differentiation of osteoblasts in the mineralized matrix, and are embedded in osteocytic lacunae (126).

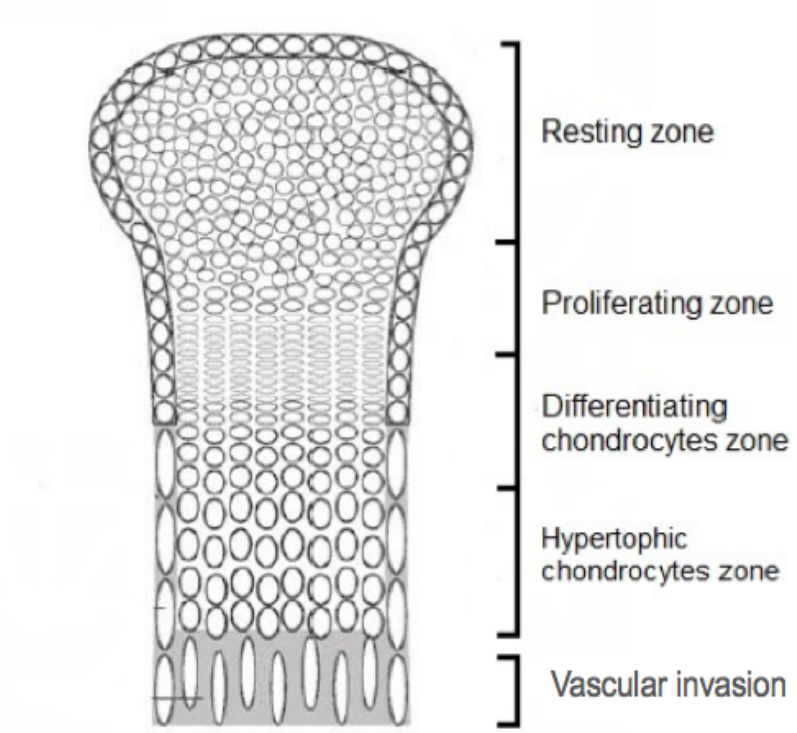

Figure 3. The growth plate in mammals.

The growth plate includes the resting zone, the proliferating zone, the differentiating chondrocytes zone and a zone of hypertrophic chondrocytes. Adapted with permission from (131) Appendix C.

1.4.1 Bone Remodeling

Bone is a metabolically-active tissue that continuously goes through remodeling processes throughout life. This is essential to maintain structure and provide the body with calcium and phosphorus when needed. Bone remodeling is a complex process involving a series of steps that are highly regulated by several local and systemic factors including hormones, cytokines, chemokines, and biomechanical stimulation (132,133). There are three phases in this process: initiation of bone resorption by osteoclasts, the transition (or reversal period) from resorption to new bone formation, and bone formation by osteoblasts (132,133).

Bone remodeling starts early in fetal life, and once the skeleton is completely formed almost all the metabolic activity in bone is dedicated to this process. The osteoclasts, osteoblasts and osteocytes closely collaborate to form the basic multicellular unit (BMU; (134). Collagenase is secreted by lining cells to digest the unmineralized matrix layer. The bone surface is then reabsorbed by osteoclast cells. The resorbed area is then reformed by osteoblast cells. Once bone formation is complete, some of the osteoblasts differentiate into lining cells (134).

The cycle of bone remodeling occurs in a specialized vascular structure called the bone remodeling compartment (BRC; (135). This compartment is created by bone-lining cells to offer a contained microenvironment for coupling and regulation of cellular activity and to avoid interference by local bone marrow growth factors. Further research on the BRC is required to completely understand the process (136).

1.4.2 Calcitriol-VDR signaling and bone homeostasis

The paramount importance of calcitriol-VDR signalling in neonatal and/or adult bone homeostasis is evidenced by the presence of rickets or osteomalacia in patients with VDDR type I and II (72,73). However, as discussed previously, the complete rescue of osteomalacia/rickets in adult *Vdr* and *Cyp27b1* null mice (34,36,39), and in humans with genetic mutations leading to absence of *VDR* or *CYP27B1*, by a diet or parenteral infusions high in calcium and phosphorus or vitamin D, indicates that these skeletal defects are primarily due to impaired intestinal calcium absorption. In support of this notion, ablation of *Vdr* from bone cells (chondrocytes, osteoblasts, or osteocytes) or ablation of *Cyp27b1* in chondrocytes, does not lead to the rachitic phenotype (33,50,137,138).

In contrast to the findings in adults and children, less is known about the role of calcitriol in fetal bone metabolism. In rat fetuses, the VDRs are expressed by day 13 of pregnancy in the condensing mesenchyme of the vertebral column and by day 17 in osteoblasts and the proliferating and hypertrophic chondrocytes (2). In normal human fetal bone, VDR was found to be expressed in the nuclei of the perichondral mesenchymal cells (osteoblastogenic cells) and in fetal cartilage cells at 10 – 15 weeks of gestation (139). These findings suggest a possible physiological role of calcitriol-VDR signalling in fetal bone homeostasis.

However, studies in fetuses of vitamin D-deficient rats and *Cyp27b1* null pigs have reported normal skeletal lengths, morphology, ash weight, mineral content and radiologic bone mineral content (31,83,98). The wild-type, $Vdr^{+/-}$, and *Vdr* null fetuses of $Vdr^{+/-}$ mothers are also indistinguishable in terms of their skeletal size, morphology, and mineral content (31). On the

other hand, *Vdr*$^{+/-}$, and *Vdr* null fetuses born from *Vdr* null mothers are smaller and weigh less than their counterparts born of *Vdr*$^{+/-}$ mothers (31). When adjusted for these size or weight differences, the ash weight and bone mineral content of offspring born from *Vdr*$^{+/-}$ mothers were no different than the offsprings of *Vdr* null mothers (31). It is only after weaning that *Vdr* null neonates develop hypocalcemia and rickets (82), and these consequences can be prevented if a diet enriched in calcium, lactose, and phosphorus is initiated prior to weaning (30,34,36,73,140,141). In contrast to the findings for fetal vitamin D-deficient rats, an 8-week vitamin D-deficient diet in guinea pigs resulted in reduced fetal body weight, bone mineral content, and increased osteoid with expansion of the hypertrophic zone of chondrocytes (142).

The most rigorous assessment of the human fetal skeleton at birth involved babies that had died of obstetrical accidents. This study compared the babies of normal mothers with babies of mothers with osteomalacia/vitamin D deficiency. The findings from this study also showed no significant between-group differences in the fetal bone calcium or phosphorus levels, ash weight and fetal skeletal morphology (143). To test the role of vitamin D supplementation in pregnancy and its effects on the fetus, several randomized clinical interventions have been done. The results of these individual interventions (144-146), as well as meta-analyses and reviews of the studies have all concluded that increased fetal values of 25OHD do not change cord blood calcium, phosphorous, or PTH, and also have no observable skeletal effects at birth (73,104,147). Taken together, these preclinical and clinical findings indicate that fetal bone homeostasis does not require calcitriol. This is due to the fact that fetal intestines aren't the route of calcium delivery and the placenta does not require calcitriol to regulate calcium transport. On the other hand, neonates do not require calcitriol until later after birth, when intestinal calcium absorption

becomes an active or calcitriol-mediated process, as opposed to the passive absorption manifested at birth.

1.5 Rationale and Hypothesis

The critical role of calcitriol in the regulation of calcium and bone metabolism in children and adults is well-established. Studies in both human and rodent models show that severe vitamin D deficiency and the genetic inability to make calcitriol (*CYP27B1* mutation or *Cyp27b1* ablation) or the genetic inability to respond to calcitriol (*VDR* mutation or *Vdr* ablation) results in reduced intestinal calcium and phosphorus absorption, hypocalcemia, hypophosphatemia, secondary hyperparathyroidism, and undermineralized bone (rickets in children and osteomalacia in adults) (6,30,73,82,140). Thus, the main function of calcitriol in children and adults appears to be regulation of the intestinal absorption of calcium and phosphorus, thereby supplying the minerals the skeleton requires.

In contrast, less is known about the role of calcitriol in regulating mineral and bone metabolism during fetal development. The widespread expression of *VDR* and/or *CYP27B1* in fetal bone cells (chondocytes, osteoblasts etc.,), placental trophoblasts and fetal kidneys indicate that calcitriol may play an important role in fetal skeletal development and mineral homeostasis (30,72,73,82). However, as discussed already, previous works involving fetuses of severely vitamin D-deficient rats, *Cyp27b1* null pigs and *Vdr* null mice have shown normal fetal serum calcium, phosphorus, skeletal morphology and ash weight (30,72,73,82). In *Vdr* null fetuses, there was an increased rate of placental calcium transport, high circulating levels of calcitriol, increased placental expression of Pthrp and Trpv6, and normal expression of S100g and Pmca1

(31,117), these findings suggest that calcitriol is not required for fetal calcium or phosphorus homeostasis and skeletal development or mineralization. Thus, calcitriol is only required after birth to regulate intestinal calcium and phosphorus absorption.

However, the incomplete absence of calcitriol in the fetuses of severely vitamin D-deficient rats and the high circulating levels of calcitriol in *Vdr* null fetuses raise other possibilities. It is plausible that the high levels of calcitriol in *Vdr* null fetuses may act through receptors rather than the known classical VDR. If so, this would explain the increased placental calcium transport and up-regulated placental expression of Pthrp and Tprv6 in the *Vdr* null fetuses. Alternatively, it is possible that calcitriol normally acts by inhibiting the placental calcium transport, such that the loss of its receptor causes increased placental calcium transport.

As already noted, the fetuses of severely vitamin D-deficient rats still have detectable levels of calcitriol (115) and *Vdr* null mice fetuses have been reported to have high circulating levels of calcitriol(31,117). Thus, the discrepancies of the existing dataset in the literature can only be resolved by studying the fetal mineral homeostasis and bone metabolism in *Cyp27b1* null fetuses, which would be completely devoid of calcitriol.

I hypothesized that calcitriol acts solely through VDR to regulate fetal bone and mineral homeostasis. Consequently, *Cyp27b1* null fetuses, when compared to their *Cyp27b1*$^{+/-}$ or wild type (WT) siblings, should have normal serum minerals, calciotropic hormone levels, skeletal morphology and mineral content, placental phosphorus transport, and relevant gene expression within the placenta and kidneys. If calcitriol inhibits placental calcium transport, then the rate of

transport and associated gene expression should increase as found in *Vdr* null fetuses. In contrast, if calcitriol acts through other receptors to regulate placental calcium transport, then *Cyp27b1* null fetuses (which lack calcitriol) may show normal or reduced placental calcium transport as compared to the increased placental calcium transport in *Vdr* null fetuses, which have high concentrations of calcitriol in their circulation.

2 MATERIAL & METHODS

2.1 *Cyp27b1* Knockout Mouse Model

Mice were obtained from Dr. René St-Arnaud (McGill University, Montréal, Quebec). A Cre-mediated excision of the *Cyp27b1* gene was performed in embryonic stem cells to deactivate the Cyp27b1 enzyme. The *Cyp27b1* null allele was created by deleting exon 8 encoding the heme binding domain and part of introns 7 and 8 of the *Cyp27b1* gene (32). The presence of hypocalcemia, hypophosphatemia, hyperparathyroidism and undetectable calcitriol levels in the serum of adult homozygous mutants confirmed the successful inactivation of the 1α-hydroxylase gene (32). Heterozygous males and females were mated and generated three genotypes: wild type with both normal *Cyp27b1* alleles ($Cyp27b1^{+/+}$), heterozygous with one normal *Cyp27b1* allele and one *Cyp27b1* allele ablated ($Cyp27b1^{+/-}$), and null with both *Cyp27b1* alleles ablated ($Cyp27b1^{-/-}$).

2.1.1 Animal Husbandry

Mice were maintained in a facility operated by Animal Care Services of Memorial University of Newfoundland, in accordance with the Canadian Council on Animal Care (CCAC). Twelve-hour light/dark cycles consisted of light from 8:00 am to 8:00 pm and darkness from 8:00 pm to 8:00 am. $Cyp27b1^{+/-}$ male and female mice were given water and a standard rodent diet (1% calcium, 0.75% phosphorus) *ad libitum*. *Cyp27b1* null and their WT sisters were maintained on the rescue diet (TekLad TD.94112; Harlan Teklad, Madison, WI) from weaning. Males were housed individually for breeding, while females were housed 3-4 per cage. All experiments were approved by the Institutional Animal Care Committee (IACC; 15-02-CK) of Memorial University of Newfoundland.

2.1.2 Mating Strategy

The present study herein involved two mating strategies. The first mating strategy involved time-mating of *Cyp27b1*$^{+/-}$ male and female mice to generate pregnancies with WT, *Cyp27b1*$^{+/-}$, and *Cyp27b1* null fetuses. The second strategy involved mating *Cyp27b1* null and WT females with *Cyp27b1*$^{+/-}$ males to generate *Cyp27b1* null and *Cyp27b1*$^{+/-}$ fetuses from *Cyp27b1* null dams and WT and *Cyp27b1*$^{+/-}$ fetuses from WT dams. This strategy allowed us to compare *Cyp27b1* null and *Cyp27b1*$^{+/-}$ fetuses from *Cyp27b1* null dams relative to that of *Cyp27b1*$^{+/-}$ and WT fetuses from WT mothers.

2.2 Genotyping

The genotypes of fetal and adult mice were identified by amplification of one target identifying the null allele and the other identifying the WT allele sequence through polymerase chain reaction (PCR) and the subsequent resolution of the gene products by gel electrophoresis. The following sections discuss the methods of various steps involved in the genotyping.

2.2.1 Animal identification and tail sample collection

Newly-weaned mice were briefly anesthetized under isoflurane (Fisher Scientific, Burlington, ON), and an ear tag containing a unique letter and number was crimped into the right ear of mice for identification purposes. Subsequently, tail samples (~0.5 cm) from each mouse were collected for genomic DNA isolation. The collected tail samples were then placed in 300 µl of cell lysis solution and 1.5 µl of Proteinase K (Qiagen Puregene® Core Kit A; Qiagen, Toronto, ON), and allowed to digest over night at 55ºC. Following tail sample collection, the tail blood was allowed to clot prior to placing the mice back in their home cages.

2.2.2 Genomic DNA Isolation

Genomic DNA was extracted from the tail samples using the Qiagen Puregene® Core Kit A, as per the manufacturer's instructions. Briefly, 100 µl of protein precipitation solution was added to tubes containing digested tail samples. Tubes were vortexed for 20 seconds on a high speed and then centrifuged for 3 minutes at 16,000 x g. The supernatant was poured into a new Eppendorf tube containing 300 µl of 2-Propanol (Fisher Scientific, Burlington, ON). The tubes were then inverted 50 times to precipitate the DNA and then centrifuged for 1 minute at 16,000 x g to pellet the DNA. The supernatant was discarded after centrifugation, and 300 µl of 70% ethanol was added to the tubes to wash the DNA pellet. The tubes were inverted again several times and centrifuged for 1 minute at 16,000 x g. Ethanol from the tubes was drained onto a piece of absorbent paper, and excess ethanol was pipetted out. The tubes were left open for 5 minutes to allow any remaining ethanol to evaporate. Subsequently, 100 µl of DNA hydration solution was added and samples were vortexed for 5 seconds. The samples were then incubated at 65ºC in a heat block for one hour to dissolve the DNA. The isolated genomic DNA was stored at 2-8ºC until further experimentation.

2.2.3 Polymerase Chain Reaction

The PCR was carried out on the genomic DNA isolated from tail samples. A three-primer system was used to amplify gene products. The forward primer was used to detect both the null and WT allele, primer 2 (reverse) was used to detect the null allele, and primer 3 (reverse) was used to detect the *Cyp27b1* WT allele (Figure 4). Reverse primers 2 and 3 is able to detect and initiate replication on the WT allele while the site for primer 3 is missing in the null allele. Therefore, the null allele can only produce a band produced by primer 1 and 2. The WT allele can produce

two different bands, between primer 1 and 2, and between primer 1 and 3. However, the band predicted by primer 1 and 3 is shorter than the one produced by primer 1 and 2, the production of the shorter band is favored. Therefore, the null allele corresponds to a 350bp product versus a 250bp product for the WT allele (Figure 4).

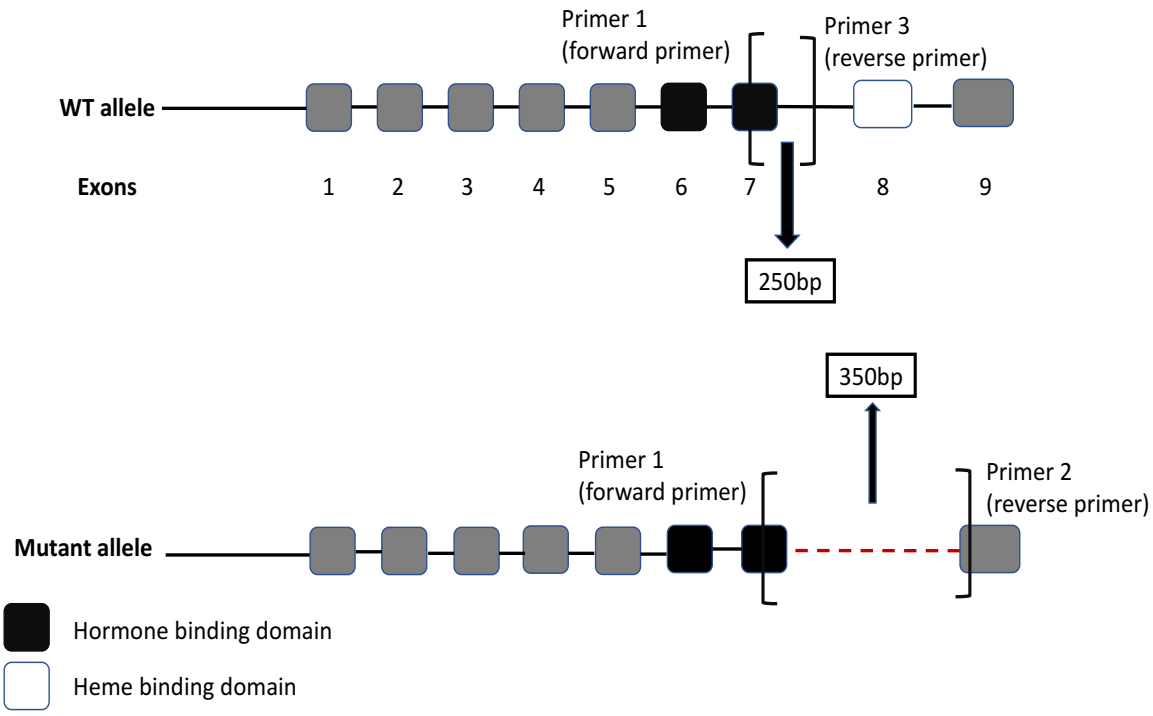

Figure 4. Schematic presentation of the alignment of primers used to genotype the *Cyp27b1* mice. A 3-primer system was used to distinguish WT, Cyp27b1$^{+/-}$ and Cyp27b1 null mice. Primer 1 detected both the WT and Cyp27b1 null allele, primer 3 detected the WT allele, and primer 2 detected the null allele.

The primer sequences are as follows:

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

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

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

The PCR cocktail contained the following: 10X PCR buffer (20 mM Tris-HCL, pH 8.4, 50 mM potassium chloride), deoxyribose nucleotide tri-phosphates (dNTPs) (0.2 mM Deoxyadenosine-5'-triphosphate (dATP) : 0.2 mM Deoxythymidine triphosphate (dTTP) : 0.2 mM Deoxycytidine triphosphate (dCTP) : 0.2 mM Deoxyguanosine triphosphate (dGTP): H_2O = 1 : 1 : 1 : 1 : 1) (LifeTechnologies, Burlington, ON), primers (*Cyp27-1, Cyp27-2, Cyp27-3*) (LifeTechnologies, Burlington, ON), 50 mM $MgCl_2$, 1 U Taq DNA Polymerase (0.02 U/ µL) (LifeTechnologies, Burlington, ON) and deionized water. 19.5 µl of PCR cocktail was aliquoted into each labeled reaction tube. 2 µl of the genomic DNA sample was added and the tubes were then placed in the PCR machine (C1000 Touch™ Thermal Cycler; Bio-Rad, Saint-Laurent, QC).

The following steps were used to amplify PCR products: Step 1: 94°C for 5 minutes to initialize the reaction, Step 2: 94°C for 30 seconds to denature the DNA, Step 3: 58°C for 30 seconds to anneal the primers, Step 4: 72°C for 30 seconds to synthesize and elongate a complementary strand, Step 5: return to Step 2 and repeat for 35 cycles for continued amplification, Step 6: 72°C for 10 minutes to elongate any remaining strands and Step 7: hold at 4°C indefinitely.

2.2.4 Gel Electrophoresis

Gel electrophoresis was performed to separate and visualize the PCR products using a 1.2% agarose gel (1.2 g agarose (LifeTechnologies, Burlington, ON) 10 ml 10× Tris-acetate-EDTA buffer (TAE; 0.12 M EDTA, 0.40 M Tris, 11.5% Glacial Acetic Acid, pH 8.0), 90 ml deionized water and 20% SYBR^{TM} Safe DNA Gel Stain (LifeTechnologies, Burlington, ON). The prepared gel was poured into a gel electrophoresis apparatus with 24-well combs and allowed to polymerize for 30 minutes. Subsequently, 4 µl of 6x loading dye (10 mM Tris, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM of 0.5 M EDTA, 1.5 M dH2O) was added to each of the 21.5 µl PCR products. Following solidification of the gel, 15 µl of the PCR product-loading dye mix was loaded into each well of the gel. The gel was run at 200 V for 20 min in a 1× TAE running buffer.

The PCR products were visualized under UV light using the Kodak Gel Doc System (Kodak, Toronto, ON), and an image was captured using Bio-Rad Image Lab version 5.1 software. The presence of a single band at 250 bp indicated the mouse was WT, a single band at 350 bp showed the mouse was *Cyp27b1* null, and the presence of both bands indicated the mouse was heterozygous (Figure 5).

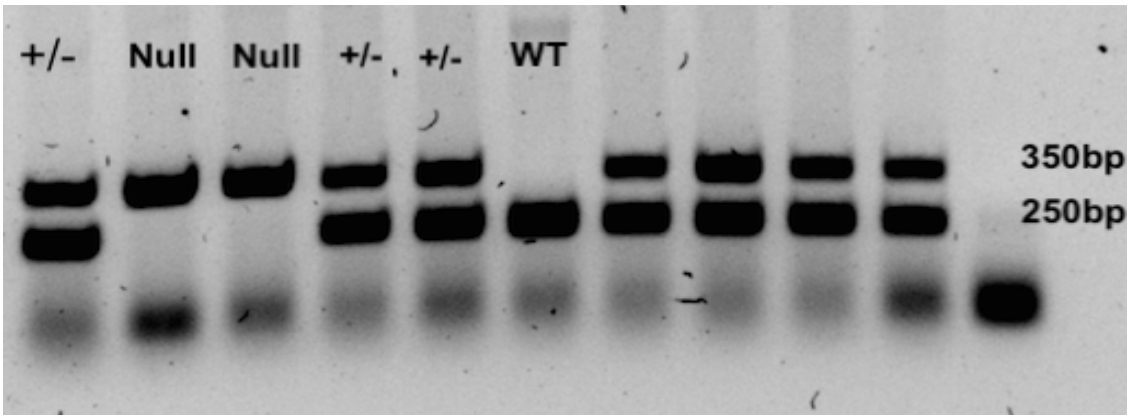

Figure 5. Representative Image of PCR products resolved using Gel Electrophoresis.

Sample PCR products were run on a 1.2% agarose gel. As indicated above, the presence of both 250-bp and 350-bp bands represents a *Cyp27b1*$^{+/-}$ mouse, the presence of a single 250-bp band indicates a WT mouse, and the presence of a single 350-bp band indicates a *Cyp27b1* null mouse.

2.3 Blood, Tissue and Amniotic Fluid collection and storage

On embryonic day 18.5 or 17.5, whole blood was collected from the tail vein of *Cyp27b1*$^{+/-}$ dams. Briefly, 1 mm was cut off the tip of the tail with a surgical blade. The tail was massaged and blood was collected into a regular capillary tube for serum collection. The animals were then anesthetized with isoflurane and euthanized by cervical dislocation. A caesarean section (C-section) was perfomed to remove the embryonic sac, and fetuses were quickly removed from the embryonic sac. The placentas were also collected from each fetus and placed into labeled Eppendorf tubes and immediately snap-frozen in liquid nitrogen and stored at -80°C.

Fetal blood was collected in a regular capillary tube by making an incision in the neck with a razor blade. Fetuses were then euthanized by decapitation. Fetal kidneys were collected from fetuses and immediately snap-frozen in liquid nitrogen and stored at -80°C. The tail samples of the fetuses were collected for genotyping and the caracasses were stored at –20°C for ashing or in scintillation vials containing 10% buffered formalin (Fisher Scientific, Burlington, ON) for bone histomorphometric assessment.

2.3.1 Serum Collection

Capillary tubes containing maternal blood were placed in an Eppendorf tube and allowed to drain and blood was left to clot for serum samples. Once clotted, the tubes were then centrifuged in a Micro-MB microcentrifuge (Fisher Scientific, Burlington, ON) for approximately 5 minutes at 16,000 x g to separate the serum from blood cells. The serum was pipetted into a new 0.6-ml tube and stored at -20°C.

Fetal whole blood was collected using 60-µl micro-hematocrit capillary tubes and centrifuged at maximum speed for 5-10 minutes. The serum was clearly separated from the blood cells. A glass cutter was used to cut the capillary tube at the point where the serum and the blood cells meet. The serum was then ejected into a 0.6-ml microcentrifuge tube using a pipet bulb and stored at -20°C.

2.3.2 Amniotic Fluid Collection

A terminal C-section was carried out on pregnant *Cyp27b1*$^{+/-}$ mothers on ED 17.5 to remove the intact uterus from the abdomen. The amniotic fluid was collected from each fetal sac using 60-µl micro-hematocrit capillary tubes. The fluid samples were then transferred to 0.6-ml microcentrifuge tubes and stored at -20°C until further experimentation.

2.4 Mineral and Skeletal Morphometric Assessment

2.4.1 Serum and Amniotic Fluid Total Calcium Measurement

The test principle of the assay is based on the formation of Arsenazo III-calcium complex (blue-purple color) as a result of a reaction between Arsenazo III and calcium. The color has a maximal absorbance at a wavelength of 650 nm and is directly proportional to the concentration of calcium in the samples. The calcium levels were assessed in both undiluted serum and amniotic fluid samples. All samples (serum and amniotic fluid) were analyzed using the Sekisui Calcium assay kit, according to the manufacturer's protocol (Sekisui, Diagnostics PEI Ltd., Charlottetown, PE). The samples were collected as described above. The amount of serum/amniotic fluid from one fetus was sufficient to provide the 10 µl volume the assay required. A spectrophotometer (Ultrospec 2000; Pharmacia Biotech, Piscataway, NJ) was used to

determine the absorbance at 650 nm. Standard solutions of known calcium concentrations provided in the kit were used to generate a standard curve and were subsequently used to determine the calcium concentrations in the unknown serum or amniotic fluid samples.

2.4.2 Serum and Amniotic Fluid Inorganic Phosphate Measurement

The principle of this assay is based on the reaction of inorganic phosphorus with ammonium molybdate in the presence of sulfuric acid to produce an unreduced phosphomolybdate complex. Following kit instructions (Sekisui Diagnostics, Charlottetown, PEI), 10 μl of undiluted serum or amniotic fluid were used to measure the concentration of inorganic phosphorus using a spectrophotometer (Ultraspec 2000, Pharmacia Biotech, Piscataway, NJ) at 340 nm. Standard solutions of known inorganic phosphate concentrations provided in the kit were used to generate a standard curve and were subsequently used to determine the inorganic phosphate concentrations in the unknown serum or amniotic fluid samples.

2.4.3 Amniotic Fluid Magnesium

Magnesium in amniotic fluid was assessed using a colorimetric assay. The magnesium test is based on a reaction between xylidyl blue-1 and magnesium ions to form a Mg-xylidyl blue complex (red). Following the procedure in the kit (Genzyme Diagnostics P.E.I. Inc., Charlottetown, PE), the results of absorbance were read at 520 nm. Amniotic fluid samples were measured undiluted using a spectrophotometer (Ultrospec 2000, Pharmacia Biotech, Piscataway, NJ) to read the absorbance at a 650-nm wavelength.

2.4.4 Bone Histomorphometry

The fetal tibias were dissected from the whole fetus and embedded in paraffin by histological services (Health Sciences Centre, St John's, NL). From these paraffin blocks, 5-µm serial sections were cut using a Leica RM 2135 microtome (Leica Biosystems Inc., Concord, ON) and fixed on slides for Von Kossa staining.

2.4.5 Von Kossa Staining

This method works by detecting inorganic phosphates and carbonates. However, calcium is the only known cation that binds to the inorganic phosphate and carbonate anions in bone tissues (148,149). In this method, the silver displaces calcium to create black deposits of silver phosphate and silver carbonate. Slides of fetal tibial sections were placed on a slide warmer (Fisher Scientific) for 30 minutes. To deparafinize and rehydrate the samples, the following steps were followed: Xylene – 2 minutes (2x), 100% Ethanol (EtOH) – 2 minutes (2x), 95% EtOH – 2 minutes, 70% EtOH– 2 minutes, 50% EtOH – 2 minutes, and distilled water – 1 minute. The slides were then incubated in a 1% aqueous silver nitrate solution and exposed directly to light for 60 minutes. Sections were then washed in distilled water three times for 2 minutes each. Following this washing, the slides were placed in a 2.5% sodium thiosulphate solution for 5 minutes. Following further washes with water, sections were then counterstained with methyl green (Sigma/Aldrich, Oakville, ON) for 2 minutes. The corners of the slides were blotted on a paper towel to remove the excess dye solution and then washed twice in 1-butanol (Fisher Scientific, Burlington, ON). The sections were then washed in xylene twice (10 seconds each time). As a final step, Permount (Fisher Scientific, Burlington, ON) was used on the slides to mount the coverslips.

2.4.6 Fetal Ash and Skeletal Mineral Assay

Carcasses of genotyped fetuses embryonic day (ED) 18.5 were placed in ceramic crucibles and then reduced to ash using an oven (Thermo Scientific Thermolyne F62735 Oval Muffle Furnace, Thermo Fisher Scientific, Walthan, MA) at 500°C for 24 hours. The ash of each fetus was carefully transferred to a piece of weighing paper and weighed on an analytical balance. The ash weight represents the total mineral content of the fetal skeleton. The ash was transferred into labelled, acid-washed 20-ml scintillation vials and stored at room temperature for mineral analysis.

Atomic Absorption Spectroscopy was used to analyze the amount of calcium and magnesium in the skeletal ash. To dissolve the ash, 253 µl of nitric acid was added to each scintillation vial and ash was left at room temperature to dissolve. After 5 days, 9.747 mL of distilled water was added to each vial for a total volume of 10 mL and a concentration of 0.2 N nitric acid. The concentration of calcium and magnesium in the ash was measured using a 2380 atomic absorption flame spectrometer (Perkin-Elmer, Walthan, MA).

To measure the phosphorous content in the fetal ash, a colorimetric Phosphorus-SL Assay Kit, described above for measuring serum phosphorous, (Sekisui Diagnostics, Charlottetown, PE) was used. All samples were diluted 1:10 in distilled water prior to measurement.

2.5 Hormone Assays

2.5.1 PTH Enzyme-Linked Immunosorbent Assay (ELISA)

The parathyroid hormone (PTH) concentrations in fetal and maternal serum samples were assessed using an Enzyme-linked ImmunoSorbent Assay (ELISA) Kit (Immunotopics Inc, San Clement, CA.). Briefly, the test principle was based on two-site ELISA methodology: The C-terminal portion is detected by an anti-rat PTH antibody attached to the well surface and ready for biotinylation capture. The N-terminal portion is recognized by a horseradish peroxidase (HRP)-labeled anti-rat antibody and forms a sandwich complex with the captured antibody. The enzymatic activity of the antibody complex bound to the well is directly proportional to the amount of PTH in the sample. 20 µl of undiluted serum was added to wells of the coated plate and the kit protocol was followed. After substrate incubation, results were read at 450 nm in a microplate spectrophotometer (Epoch; Biotek, Winooski, VT) and analyzed using Gen5 software (Version 2.04.11, BioTek Instruments Inc, Winsooki, VT).

2.5.2 Calcitriol

On ED 18.5, fetal serum calcitriol was measured using an ELISA kit, which is a kit for detecting human calcitriol that cross-reacts with mouse calcitriol (Immunodiagnostic Systems, Inc, Gaithersburg, MD). The two-day experiment involved an initial immunoextraction to purify 1,25OHD in serum and then a subsequent ELISA to measure calcitrol in the serum samples. The minimum sample size needed for this assay was 120 µl. The maximum amount of fetal serum we could get was ~35 µl. Therefore, three or four samples were pooled together to reach the volume needed for 1 sample. The test was based on binding competitions between either free 1,25OHD or 1,25OHD linked to biotin for a highly-specific sheep anti-1,25OHD binding site. The amount

of complex biotin bound to the anti-sheep antibody was inversely proportional to the concentration of 1,25OHD. The detection limit of the assay was 6 pmol/L. Absorbance was measured at 450 nm using a microplate spectrophotometer with Gen5 software (Version 2.04.11, BioTek Instruments Inc, Winsooki, VT).

2.5.3 25-hydroxyvitamin D and 24,25-dihydroxyvitamin D

Sera from *Cyp27b1* null, *Cyp27b1*$^{+/-}$ and WT fetuses was sent to Dr. Glenville Jones's laboratory (Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON) to measure two forms of vitamin D (25OHD and 24,25$(OH)_2$D). 25OHD and 24,25-dihydroxyvitamin D were measured using liquid chromatography-tandem mass spectrometry (LC–MS/MS). The assay methods and procedures are similar to those published previously (150).

2.5.4 Serum Fibroblast Growth Factor 23 (FGF23)

Fetal serum FGF23 was analyzed using an FGF23 ELISA kit (Kainos Laboratories, Tokyo, Japan). The principle of the assay is based on two-step ELISA. The surface of the well contains immobilized FGF-23 antibodies to capture FGF23. A sandwich complex forms from the first antibody and HRP-labeled FGF23 antibody. The enzymatic activity of the antibody complex bound to the well is directly proportional to the amount of FGF23 in the sample. The detection limit of the assay was 3 pg/ml. The absorbance at 450 nm was measured using a microplate spectrophotometer with Gen5 software (Version 2.04.11, BioTek Instruments Inc, Winsooki, VT).

2.6 Calcium and Phosphorus Placental Transport

Placental calcium transport was measured using a previously published protocol (Kovacs, et al. 1996). On day 18.5 of gestation, *Cyp27b1* heterozygous dams were briefly anesthetized with isoflurane. 100 µl of a solution containing 50 µCi (1.85 MBq) of ^{45}Ca and 50 µCi (1.85 MBq) of ^{51}Cr-EDTA in 0.9% saline was administered by intercardiac injection. After 5 minutes, the mother was euthanized by cervical dislocation and a C-section was carried out to remove the fetuses. Each fetus was quickly removed from its amniotic sac and a tail snip was taken for genotyping. The fetus was then placed in a 12-mm × 75-mm disposable round bottom polystyrene culture test tube (Fisher Scientific, Burlington, ON) and forceps were used to pith the brain. Tubes were capped and the ^{51}Cr-EDTA activity in each fetus was determined using a 1480 WIZARD™ 3" automatic gamma counter (Perkin Elmer, Guelph, ON). 1 ml of ScintiGest™ (Fisher Scientific, Burlington, ON) was added to the tube and the tube was vortexed to loosen the fetus from the walls of the tube. The contents of the tube were poured into disposable 20-ml scintillation vials (Fisher Scientific, Burlington, ON) and an additional 9 ml of ScintiGest™ was then added to each vial. The scintillation vials were then incubated in an isotherm oven (Fisher Scientific, Burlington, ON) at 55°C for 24 hours to solubilize. The vials were vortexed several times during this incubation. Once the fetuses were completely solubilized, 10 ml of scintillation fluid (ScintiVerse®; Fisher Scientific, Burlington, ON) and 5 drops of glacial acetic acid (Fisher Scientific, Burlington, ON) were added. Each vial was then covered with aluminum foil and placed in the dark for 24 hours to reduce bioluminescence. The activity of ^{45}Ca in each fetus was then measured by an LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Montreal, QC). After measuring the activity of each isotope in each fetus, the ratio of ^{45}Ca to ^{51}Cr-EDTA activity for each fetus was used to calculate the rate of

calcium transfer. The results were then normalized to the mean value of the heterozygotes within each litter.

The placental phosphorus transport experiment was adapted from the previously-described Ca^{2+} transport experiment(74). The experiment was performed following the same procedure of placental calcium transport. On ED 18.5, pregnant *Cyp27b1* heterozygous dams were briefly anesthetized under isoflurane and injected with 1 ml consisting of 50 μCi (1.85 MBq) of ^{32}P and 50 μCi (1.85 MBq) of ^{51}Cr-EDTA in 0.9% saline intra-cardially. Following the same steps as the placental calcium transport experiment, the activity of each isotope was determined in each fetus. The ratio of ^{32}P to ^{51}Cr-EDTA activity for each fetus was used to calculate the phosphorus transfer. The ratios were then normalized to the mean value of the heterozygote fetuses within each litter.

2.7 Gene Expression Changes in the Placenta and Fetal Kidneys

The expression of calcium and phosphorus relevant genes was determined using quantitative real-time RT-PCR (qRT-PCR) in the placenta and fetal kidneys.

2.7.1 RNA Extraction

Total RNA was extracted from placentas or both kidneys using the RNeasy Midi and RNeasy Mini Kit (Qiagen, Toronto, ON), respectively. The RNA quality and quantity were analyzed using the Agilent 2100 BioAnalyzer (Agilent Technologies, Inc., St Laurent, QC) for qPCR analysis.

2.7.2 Complementary DNA (cDNA) Synthesis

A Superscript III First-strand Synthesis System (LifeTechnologies, Burlington, ON) was used for cDNA synthesis. Following the protocol in the kit, 2 µg of RNA was used for cDNA synthesis. The synthesis process followed these steps: 25°C for 10 minutes, 37°C for 2 hours, 85°C for 5 minutes, and 4°C indefinitely.

2.7.3 Real-Time Quantitative RT-PCR

The gene expression in the placenta and kidneys was analyzed using TaqMan® gene expression primer/probe sets and universal mastermix from Applied Biosystems (LifeTechnologies, Burlington, ON). The primer design consisted of MGB primers with FAM dye at the 5' end and a non-fluorescent quencher at the 3' end. Primer/probe sets for the following genes were used: *Cyp27b1*, *Cyp24a1, Calbindin-D9k (S100g), Ca^{2+}-ATPase (Pmca1), Trpv6, Pthrp, Klotho*, *NaPi2a*, *b* and *c*, and *Fgf23*. Following the protocol instructions in the kit, all samples were analyzed in triplicate. The C_T (threshold cycle value) was used as a gene expression indicator and GAPDH was used as an endogenous control. The real-time quantitative running program was as follows: Step 1: 50°C for 2 minutes for UNG incubation, Step 2: 95°C for 20 seconds for polymerase activation, Step 3: 95°C for 1 second for denature, Step 4: 60°C for 20 seconds for annealing, and Step 5: Back to Step 3 and repeat 40 times. All samples were performed in triplicate with a reaction volume of 20 µl.

2.8 Statistical Analysis

All data were analyzed using StatPlus 5.8.3.8 for Macintosh (AnalystSoft Inc, Walnut, CA). The between-group differences among means were analyzed using one-way analysis of variance

(ANOVA) followed by a Tukey-Kramer post-hoc test. Sample sizes are indicated in parentheses and biochemical data are shown with ± standard error of the mean (SEM). The qPCR data were analyzed using the $2^{-\Delta\Delta C}$T method and presented as mean± SD (151).

3 RESULTS

The validity of the *Cyp27b1* knockout mice as a rodent model of pseudo-vitamin D-deficiency is established by the presence of hypocalcemia, hyperparathyroidism, hypophosphatemia and undetectable levels of calcitriol (32). The present study investigated the effects of fetal absence of *Cyp27b1* on fetal mineral homeostasis, fetal-placental mineral transfer, and fetal skeletal morphometric features. Herein, the study assessed these parameters in *Cyp27b1* mutants born from *Cyp27b1*$^{+/-}$ dams. A preliminary dataset was also collected from *Cyp27b1* mutants born from *Cyp27b1* null dams.

3.1 *Cyp27b1* NULL FETUSES FROM *Cyp27b1*$^{+/-}$ MOTHERS

3.1.1 Serum Mineral and Hormone Homeostasis

The concentrations of total calcium and phosphorous were analyzed in serum collected on ED18.5 from fetuses of *Cyp27b1* $^{+/-}$ male and female mating. *Cyp27b1* null fetuses had normal levels of serum calcium when compared to their *Cyp27b1*$^{+/-}$ and WT littermates (Figure 6A). Similarly, there was no significant difference in the serum phosphorus levels between *Cyp27b1* null fetuses relative to that of WT and *Cyp27b1*$^{+/-}$ littermates (Figure 6B).

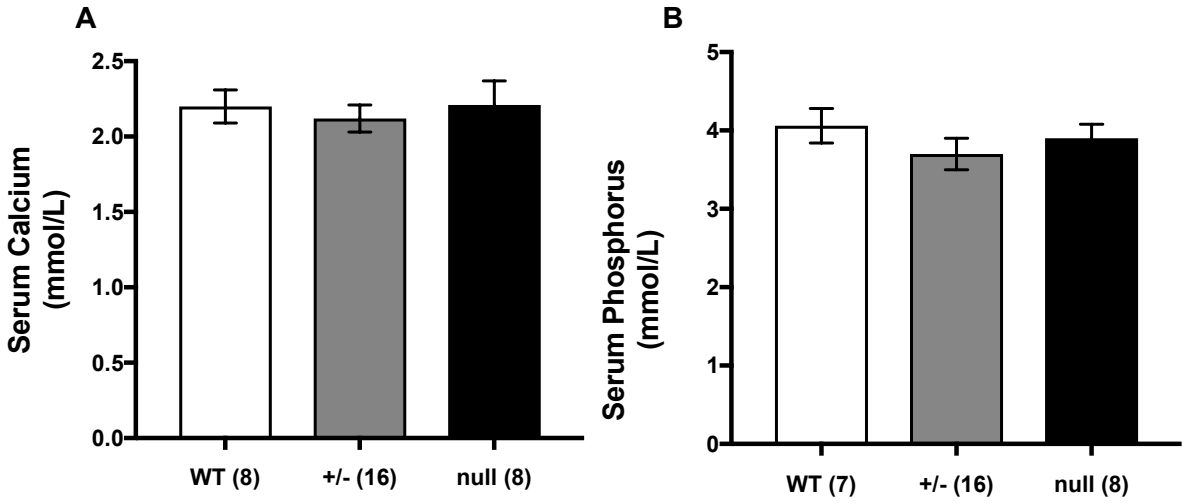

Figure 6. Serum calcium and phosphorous in fetuses obtained from *Cyp27b1*$^{+/-}$ dams.

There were no between-group differences in the serum (A) calcium and (B) phosphorous levels of the *Cyp27b1* null, *Cyp27b1*$^{+/-}$ and WT fetuses obtained from *Cyp27b1*$^{+/-}$ dams. The numbers in parentheses indicate the number of fetuses included in the study. Means reported ± SEM.

Calciotropic and phosphoric hormones such as PTH and FGF23 are known to be vital mediators of mineral and bone metabolism(72). Herein, the changes in the levels of these hormones were assessed in the fetal serum to determine the functional status of calcitriol. The levels of PTH in the fetal serum were assessed using an ELISA kit. Our findings showed no significant ($p>0.05$) differences in the serum levels of PTH in the *Cyp27b1* null fetuses compared to WT and *Cyp27b1*$^{+/-}$ fetuses born from the *Cyp27b1*$^{+/-}$ dams (Figure 7A). Likewise, similar observations were found for *Cyp27b1* null fetuses, relative to that of their WT and *Cyp27b1*$^{+/-}$ littermates, in the serum levels of FGF23 (Figure 7B).

3.1.2 Amniotic Fluid Mineral Homeostasis

The modulation of calcitriol levels has been shown previously to have a significant impact on post-natal renal mineral homeostasis (73). Hence, we assessed the levels of calcium, phosphorous and magnesium in the amniotic fluid as it is mainly composed of fetal urine. The *Cyp27b1* null fetuses born from *Cyp27b1*$^{+/-}$ mothers had similar levels of calcium, phosphorous and magnesium compared to that of WT and *Cyp27b1*$^{+/-}$ fetuses born in the same litter (Figure 8A, B and C).

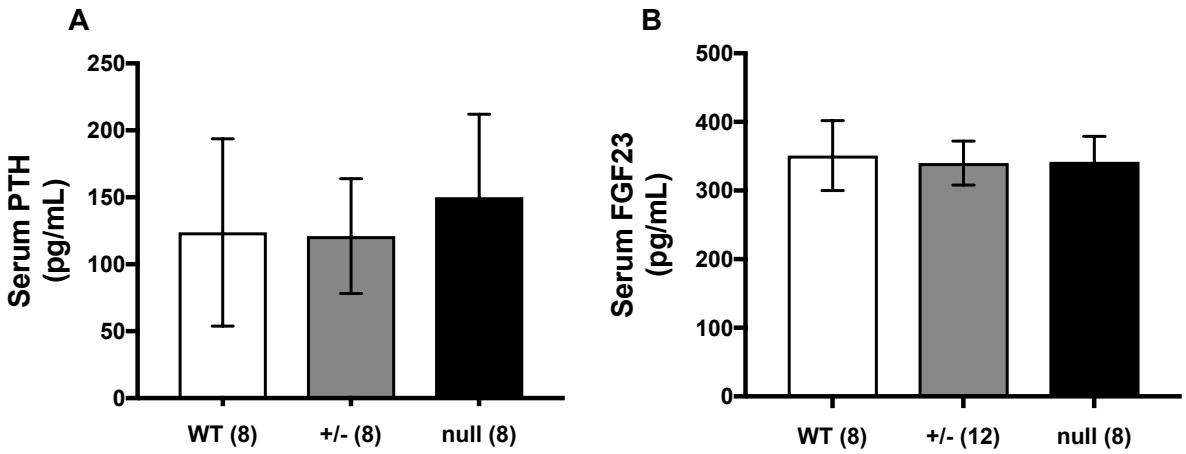

Figure 7. Serum levels of PTH and FGF23 hormones in fetuses (ED 18.5) obtained from *Cyp27b1*$^{+/-}$ mothers.

There were no differences in the serum levels of (A) PTH and (B) FGF23 in the *Cyp27b1* mutant fetuses born from *Cyp27b1*$^{+/-}$ dams. The numbers in parentheses indicate the number of fetuses studied. Means reported ± SEM.

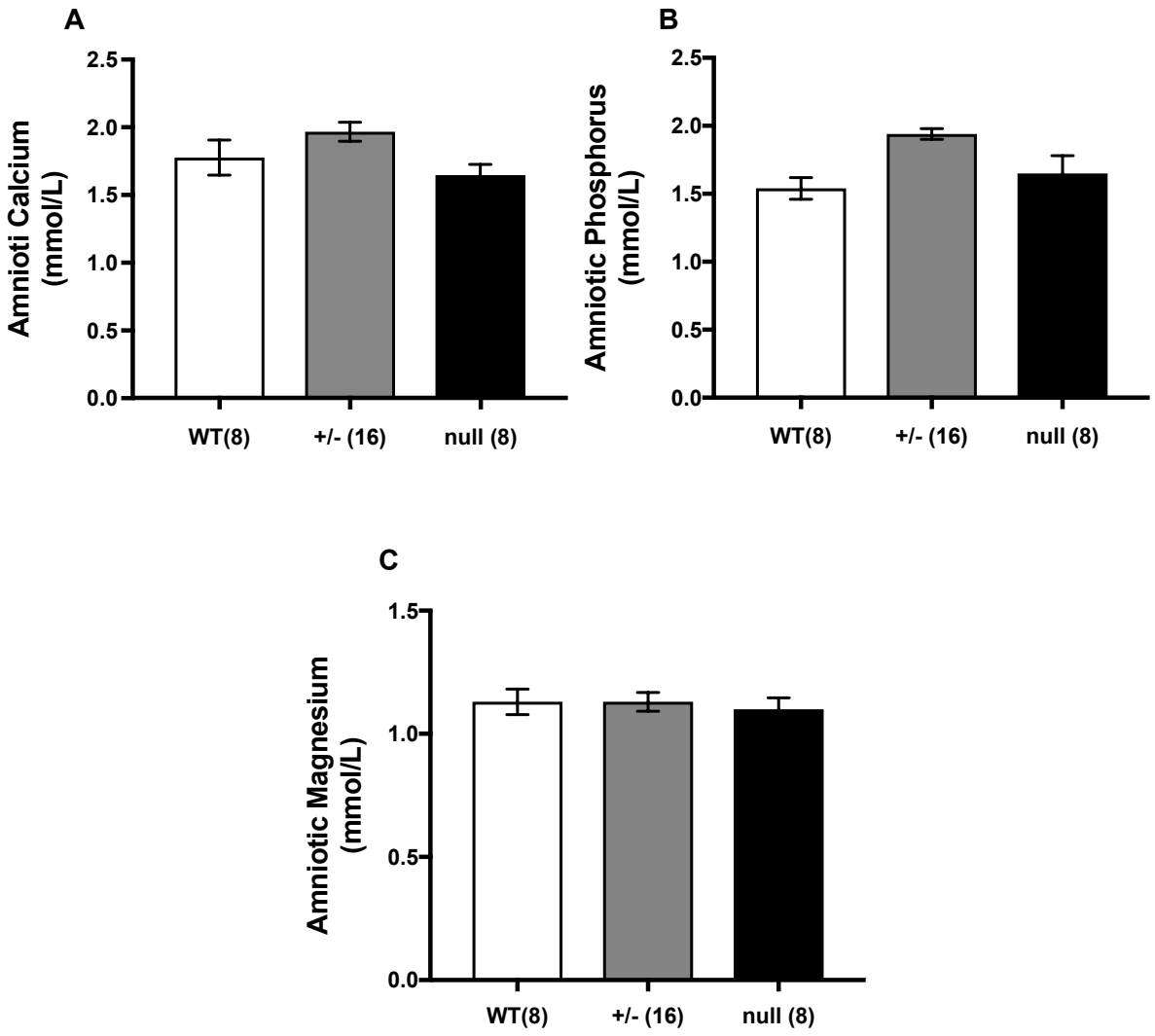

Figure 8. The levels of calcium, phosphorous and magnesium in the amniotic fluid (ED 17.5) of fetuses obtained from *Cyp27b1*$^{+/-}$ mothers.

The levels of (A) calcium, (B) phosphorous and (C) magnesium in the amniotic fluid of *Cyp27b1* null fetuses did not significantly differ from their WT and *Cyp27b1*$^{+/-}$ littermates. The numbers in parentheses indicate the number of fetuses studied. Means reported ± SEM.

3.1.3 Placental transfer of calcium and phosphorous

The impact of the deletion of the *Cyp27b1* gene on the placental transfer of calcium and phosphorus was assessed. There was no difference in placental calcium transport between *Cyp27b1* null and *Cyp27b1*$^{+/-}$ fetuses or between *Cyp27b1*$^{+/-}$ and WT fetuses (Figure 9A). Likewise, there was no difference in the placental transport of phosphorous in the *Cyp27b1* null fetuses compared to that of *Cyp27b1*$^{+/-}$ and WT (Figure 9B).

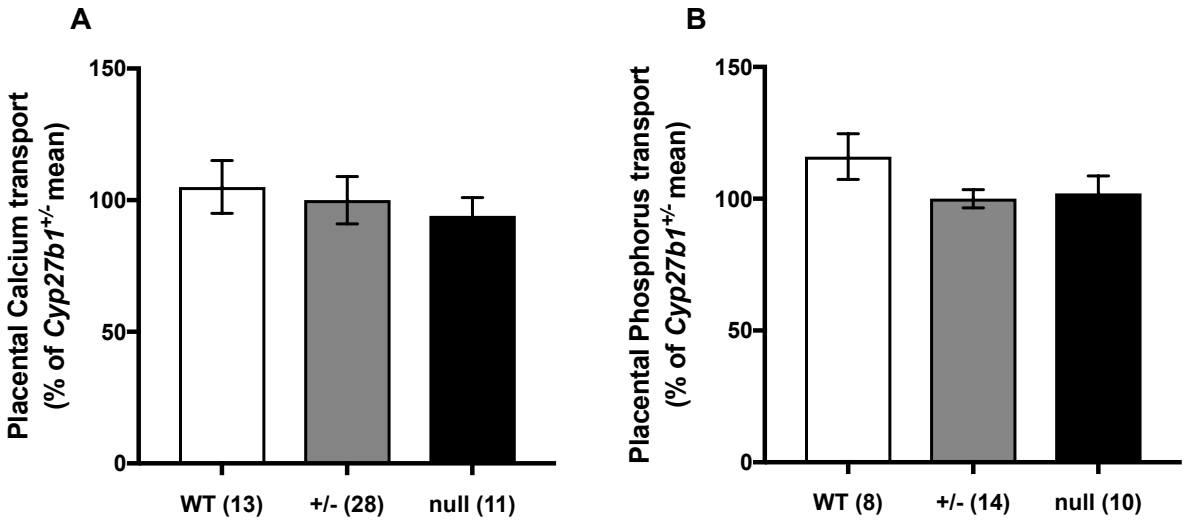

Figure 9. Placental calcium and phosphorous transfer.

The ratio of the (A) ^{45}Ca to ^{51}Cr-EDTA, and (B) ^{32}P to ^{51}Cr-EDTA radioactivity was measured in each fetus at 5 minutes after maternal intracardiac injection of the isotopes to measure the relative transfer of (A) calcium and (B) phosphorus across the placenta in the *Cyp27b1* mutant fetuses from the *Cyp27b1*$^{+/-}$ mother. The values are normalized to the mean of the *Cyp27b1*$^{+/-}$ fetuses, set at 100%, to allow comparison between the WT and *Cyp27b1* null fetuses. There was no statistically significant difference in the placental transfer of calcium or phosphorus across all three genotypes. The number in parentheses represents the number of fetuses studied. Means reported ± SEM.

3.1.4 Fetal Skeletal Morphology and Mineral Content

3.1.4.1 Bone Morphology

To investigate the skeletal mineralization and bone development in the *Cyp27b1* mutant fetuses, fetal tibias were sectioned and stained using Von Kossa stain. Herein, qualitative analysis of the stained fetal tibial bone sections showed normal distribution of mineral in both WT and *Cyp27b1* null fetuses, suggesting that the amount of mineral present in *Cyp27b1* null fetal bones did not differ from that of their WT siblings (Figure 10). In addition, the overall cellular morphology and growth plates were normal in *Cyp27b1* null fetuses compared to WT siblings (Figure 10).

3.1.4.2 Fetal ash weight and mineral content

Since the Von Kossa staining suggested normal mineral content in the *Cyp27b1* null compared to WT siblings, the next step was to quantitatively confirm these results. To do this, whole bodies of fetuses were reduced to ash in a furnace to determine the ash weight, which reflects the mineral content of bone. There was no statistically significant difference in ash weight between the WT, *Cyp27b1*$^{+/-}$ and Null fetuses born from *Cyp27b1*$^{+/-}$ dams (Figure 11A). Similar ash calcium content was observed in the WT, *Cyp27b1*$^{+/-}$ and *Cyp27b1* null fetuses born to *Cyp27b1*$^{+/-}$ mothers (Figure 11B). Likewise, both the magnesium (Figure 11C) and phosphorus (Figure 11D) contents in the skeletal ash of the *Cyp27b1* null fetuses did not differ significantly from that of the corresponding values in the WT and *Cyp27b1*$^{+/-}$ fetuses.

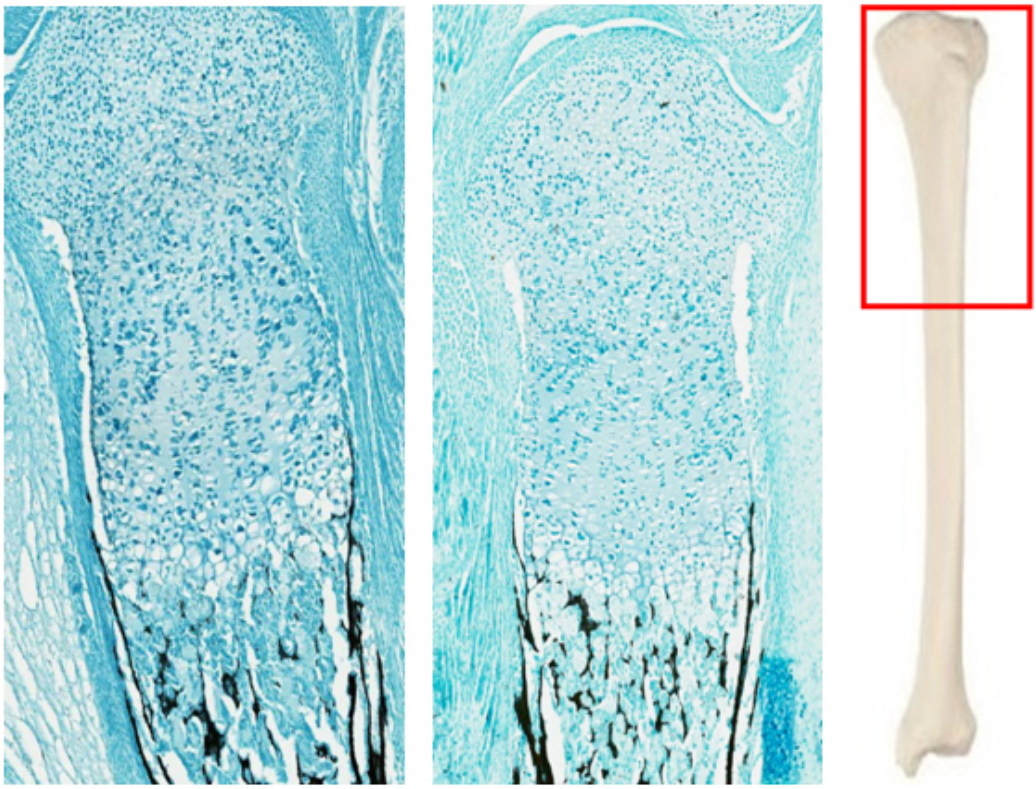

Figure 10. Von Kossa staining of tibias and growth plates that have been counter-stained with methyl green.

WT and *Cyp27b1* null fetuses (ED 18.5) were obtained from *Cyp27b1*$^{+/-}$ mothers. Representative von Kossa-stained WT and *Cyp27b1* null fetal tibial sections show normal lengths, cellular morphology in the chondrocytic and bone compartments and mineral content.

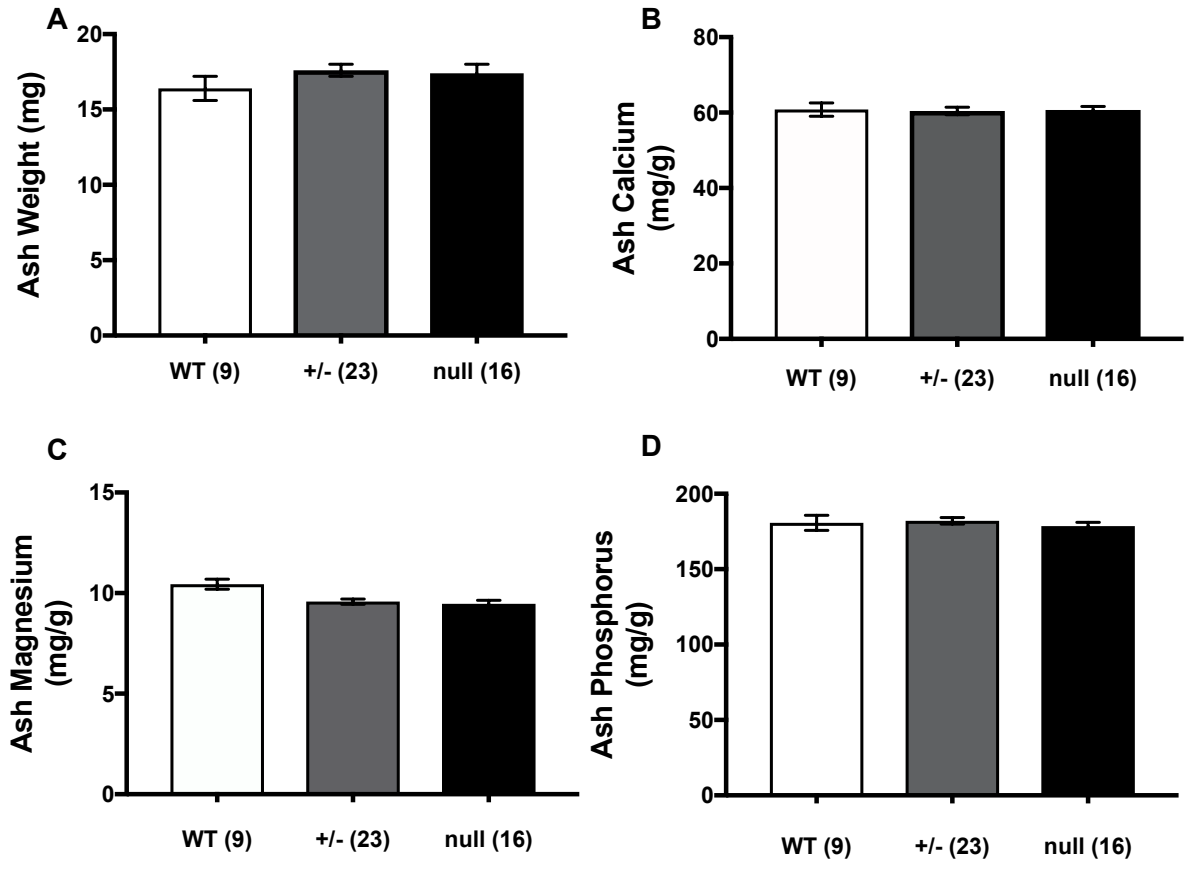

Figure 11. Fetal ash weight and mineral contents (ED 18.5).

The whole bodies of the *Cyp27b1* mutant fetuses were reduced to ash to assess the (A) weight of the ash and mineral contents of (B) calcium, (C) Magnesium and (D) Phosphorous. There were no between-group differences in any of the parameters assessed herein. The number in parentheses represents the number of fetuses studied. Means reported ± SEM.

3.1.5 Relative expression of Calciotropic and Phosphotropic genes in the placenta and fetal kidneys

Changes in gene expression of several calciotropic and phosphotropic genes in the placenta and fetal kidneys of the WT and *Cyp27b1* null fetuses were determined using qRT-PCR. Relevant genes were measured in the placenta and in fetal kidneys.

The expression of *Cyp27b1* mRNA was not absent but was significantly lower in the *Cyp27b1* null placenta compared to that of WT (Table 1). This observation is likely confounded due to the presence of maternal cells in the placenta. The absence of *Cyp27b1* mRNA in the fetal kidneys of *Cyp27b1* null fetuses confirms the successful deletion of this gene (Table 2). Interestingly, qPCR showed no significant changes in the expression of calciotropic and phosphotropic genes in the placenta and fetal kidneys assessed herein (Table 1 and 2). However, there was a significant decrease in the *Pthrp* gene expression in the *Cyp27b1* null placentas and *Cyp24a1* fetal kidneys, respectively, compared to WT fetuses (Table 1 and 2).

Table 1. Gene Expression by qPCR in *Cyp27b1* null placentas relative to WT collected from *Cyp27b1*$^{+/-}$ mothers (ED 18.5). Tests were performed in triplicate and normalized to a GAPDH control (n=5).

Gene Symbol	*Cyp27b1* null vs. WT (Fold change ± SD)	p-value
Cyp27b1	0.459 ± 0.191 vs. 1.000 ± 0.449	<0.001
Cyp24a1	1.249 ± 1.058 vs. 1.000 ± 0.982	NS
Trpv6	1.018 ± 0.291 vs. 1.000 ± 0.195	NS
PthrP	0.645 ± 0.494 vs. 1.000 ± 0.399	<0.001
S100	1.084 ± 0.131 vs. 1.000 ± 0.445	NS
Pmcab1	0.976 ± 0.055 vs. 1.000 ± 0.177	NS
Klotho	1.073 ± 0.150 vs. 1.000 ± 0.305	NS
FGF23	1.408 ± 0.809 vs. 1.000 ± 0.379	NS
Napi2a	1.123 ± 0.289 vs. 1.000 ± 0.348	NS
Napi2b	1.068 ± 0.433 vs. 1.000 ± 0.449	NS
Napi2c	1.109 ± 2.091 vs. 1.000 ± 1.575	NS

***NS – Non-significant**

Table 2: Gene Expression by qPCR in *Cyp27b1* null fetal kidneys relative to WT collected from *Cyp27b1*$^{+/-}$ mothers (ED 18.5). Tests were performed in triplicate and normalized to a GAPDH control (n=5).

Gene Symbol	*Cyp27b1* null vs. WT (Fold change ± SD)	p-value
Cyp27b1	Absent expression in null	N/A
Cyp24a1	0.033 ± 0.036 vs. 1.000 ± 0.752	0.02
Trpv6	1.013 ± 0.158 vs. 1.000 ± 0.102	NS
S100G	0.925 ± 0.136 vs. 1.000 ± 0.162	NS
Pmcab1	1.104 ± 0.076 vs. 1.000 ± 0.092	NS
NaPi2a	1.059 ± 0.183 vs. 1.000 ± 0.113	NS
NaPi2b	1.014 ± 0.220 vs. 1.000 ± 0.195	NS
NaPi2c	1.065 ± 0.282 vs. 1.000 ± 0.233	NS

***NS = Non-significant; **N/A = Undetectable**

3.1.6 Fetal Serum calcitriol, 25OHD and 24,25OH_2D

The inactivation of the *Cyp27b1* gene has been previously shown to affect the levels of calcitriol such that adult *Cyp27b1* null mutants had undetectable blood calcitriol levels. Since our experiments so far showed no significant differences in the serum and/or skeletal mineral homeostasis and/or morphology, we hypothesized that the deletion of the *Cyp27b1* gene should make calcitriol undetectable in the *Cyp27b1* null fetuses. Calcitriol was not analyzed until this point because calcitriol measurement required a high volume of serum sample as mentioned in the methods section. Since calcitriol levels can also affect the levels of its precursor 25-hydroxyvitamin D_3 ($25OHD_3$) and metabolite 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$), we determined the fetal serum levels of these metabolites as well.

The serum calcitriol levels in the *Cyp27b1*$^{+/-}$ dams were 604.7±127.8 pmoml/L (Figure 11A). Although there was a significant reduction in the serum calcitriol levels of *Cyp27b1* null fetuses (171.6±40.5 pmol/L) compared to that of WT (381.8±92.0) and *Cyp27b1*$^{+/-}$ (252.1±37.1) fetuses born from *Cyp27b1*$^{+/-}$ dams (Figure 12A). Calcitriol wasn't undetectable and was well above the detected levels observed previously in *Cyp27b1* null adult mice (12.1 pmol/L; (152). This contradicted our hypothesis that the levels of serum calcitriol in the *Cyp27b1* null fetuses would be undetectable. A possible explanation to this paradoxical observation is that the calcitriol may have crossed the placenta from the *Cyp27b1*$^{+/-}$ dams to the *Cyp27b1* null fetuses. Investigation of the fetal serum levels in the *Cyp27b1* null, *Cyp27b1*$^{+/-}$ and WT fetuses for 25OHD and $24,25(OH)_2D$ (Figure 12B and Figure 12C, respectively) found no significant between-group differences.

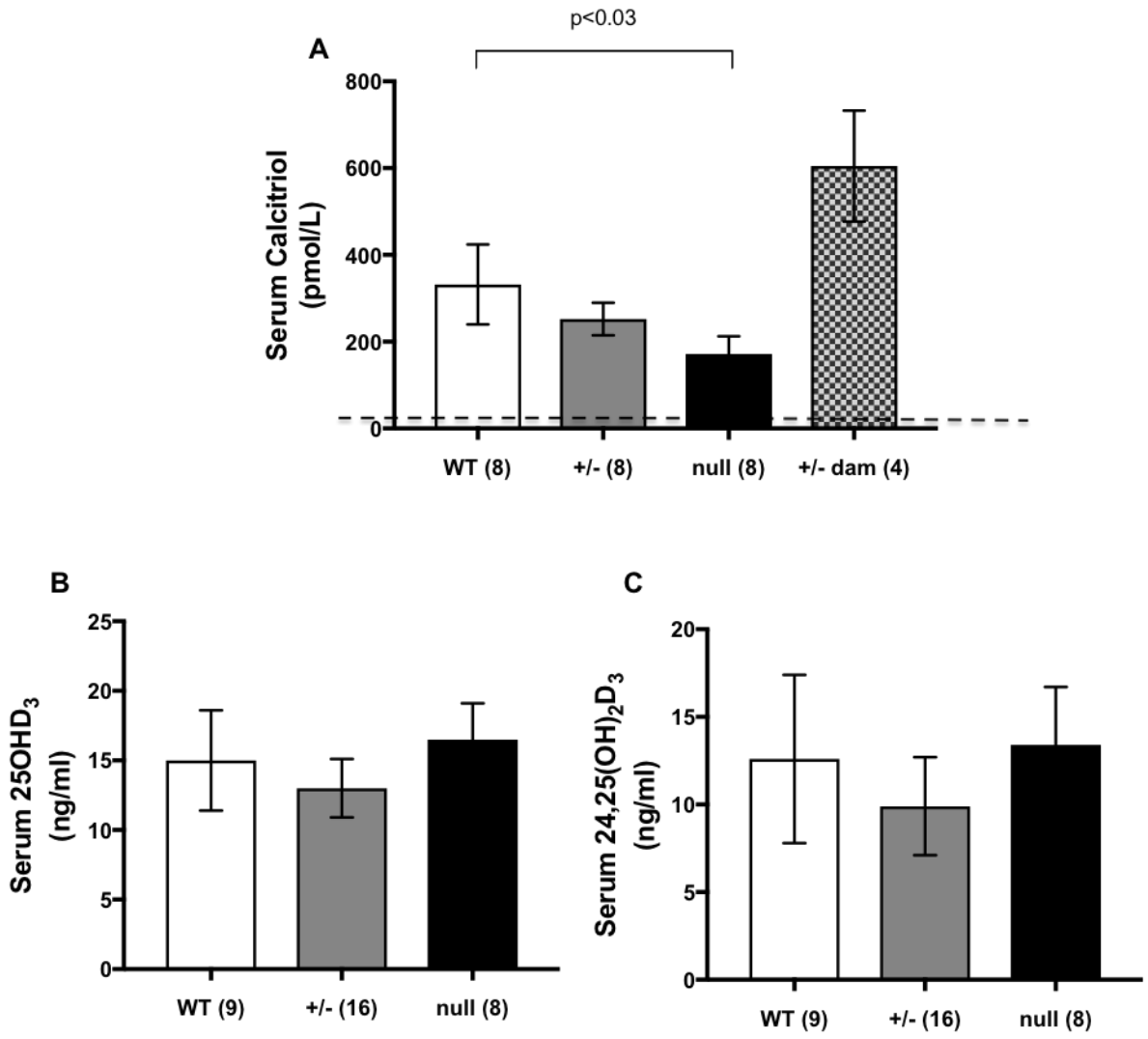

Figure 12. Serum levels of calcitriol, 25OHD and 24,25OH_2D in *Cyp27b1* mutant fetuses born from *Cyp27b1*$^{+/-}$ dams.

There was a significant reduction in the serum levels of calcitriol in the *Cyp27b1* null fetuses compared to *Cyp27b1*$^{+/-}$ and WT fetuses (A) but the values remained well above the detected levels observed in *Cyp27b1* null adult mice (dashed line). In contrast, there were no between-group differences in the serum levels of (B) 25OHD_3 and (C) 24,25OH_2D_3. The number in parentheses represents the number of fetuses studied. Means reported ± SEM.

3.2 *Cyp27b1* NULL FETUSES FROM *Cyp27b1* NULL MOTHERS: Pilot Study

In order to determine the possibility of transplacental transfer of calcitriol from mother to fetus, a preliminary study was done to study the changes in fetal mineral homeostasis of *Cyp27b1* mutant mice born from *Cyp27b1* null mothers. Data herein show that *Cyp27b1* null fetuses born of *Cyp27b1* null mothers exhibited normal serum calcium levels compared to their $Cyp27b1^{+/-}$ siblings. However, the mean levels of both were significantly increased above WT and $Cyp27b1^{+/-}$ fetuses from WT mothers (Figure 13A). Similarly, there were no differences in the serum levels of phosphorous in *Cyp27b1* null fetuses from *Cyp27b1* null mothers when compared to their $Cyp27b1^{+/-}$ littermates or to WT and $Cyp27b1^{+/-}$ fetuses from WT mothers (Figure 13B). Comparison of the fetal ash weight of *Cyp27b1* null fetuses born to *Cyp27b1* null mothers with that of their $Cyp27b1^{+/-}$ siblings or $Cyp27b1^{+/-}$ and WT fetuses from WT dams showed no significant differences (Figure 14A). Likewise, no significant differences were observed in the fetal skeletal levels of calcium (Figure 14B), phosphorus (Figure 14C) and magnesium (Figure 14D) between the *Cyp27b1* null and $Cyp27b1^{+/-}$ fetuses born from *Cyp27b1* null dams and $Cyp27b1^{+/-}$ and WT fetuses born from WT dams.

Due to time limitations and scope of my MSc project, further investigation of the skeletal morphology, the serum levels of calcitriol, PTH and FGF23 and relevant genes in the placenta and kidneys of the *Cyp27b1* mutant fetus born to *Cyp27b1* null dams was investigated by another MSc student. Our collective results have now been published together (153).

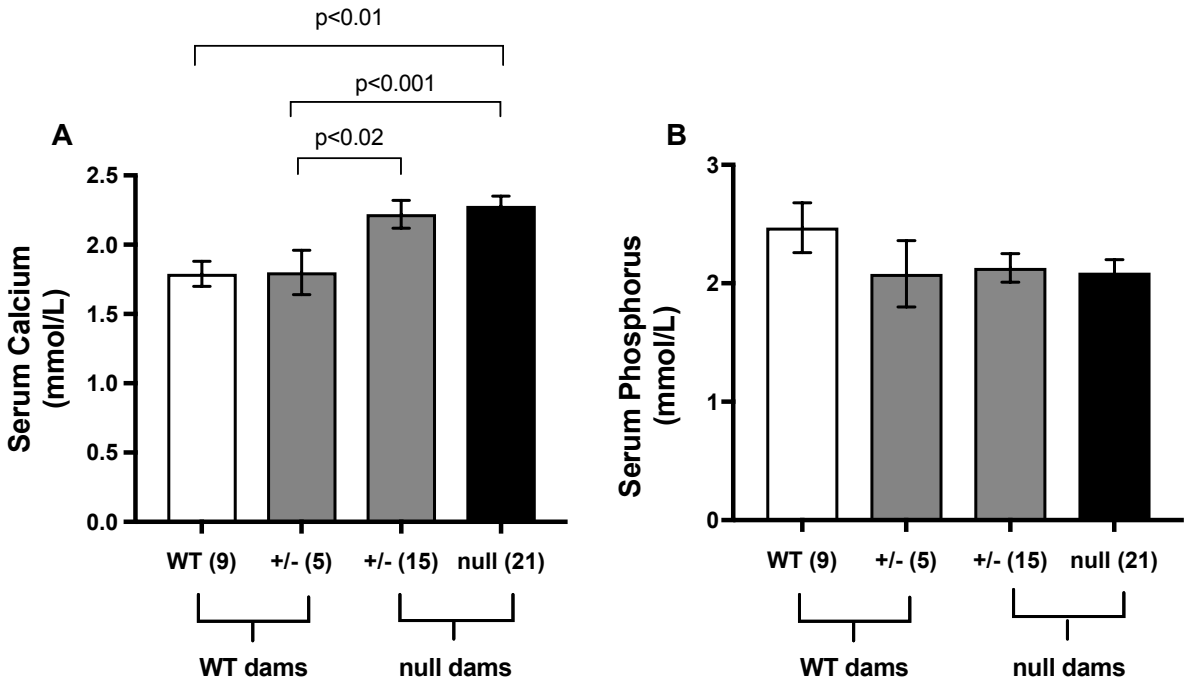

Figure 13. Serum calcium and phosphorus in fetuses obtained from *Cyp27b1* null and WT dams.

There were no significant differences in the serum levels of calcium (A) in the *Cyp27b1* null fetuses born from *Cyp27b1* null dams relative to that of *Cyp27b1*$^{+/-}$ littermates. However, both were significantly higher than those in the WT and *Cyp27b1*$^{+/-}$ fetuses of WT mothers. Serum phosphorus (B) was similar in *Cyp27b1* null fetuses born from *Cyp27b1* null dams compared to *Cyp27b1*$^{+/-}$ littermates and to the WT and *Cyp27b1*$^{+/-}$ fetuses of WT mothers. The numbers in parentheses represent the number of fetuses studied. Means reported ± SEM.

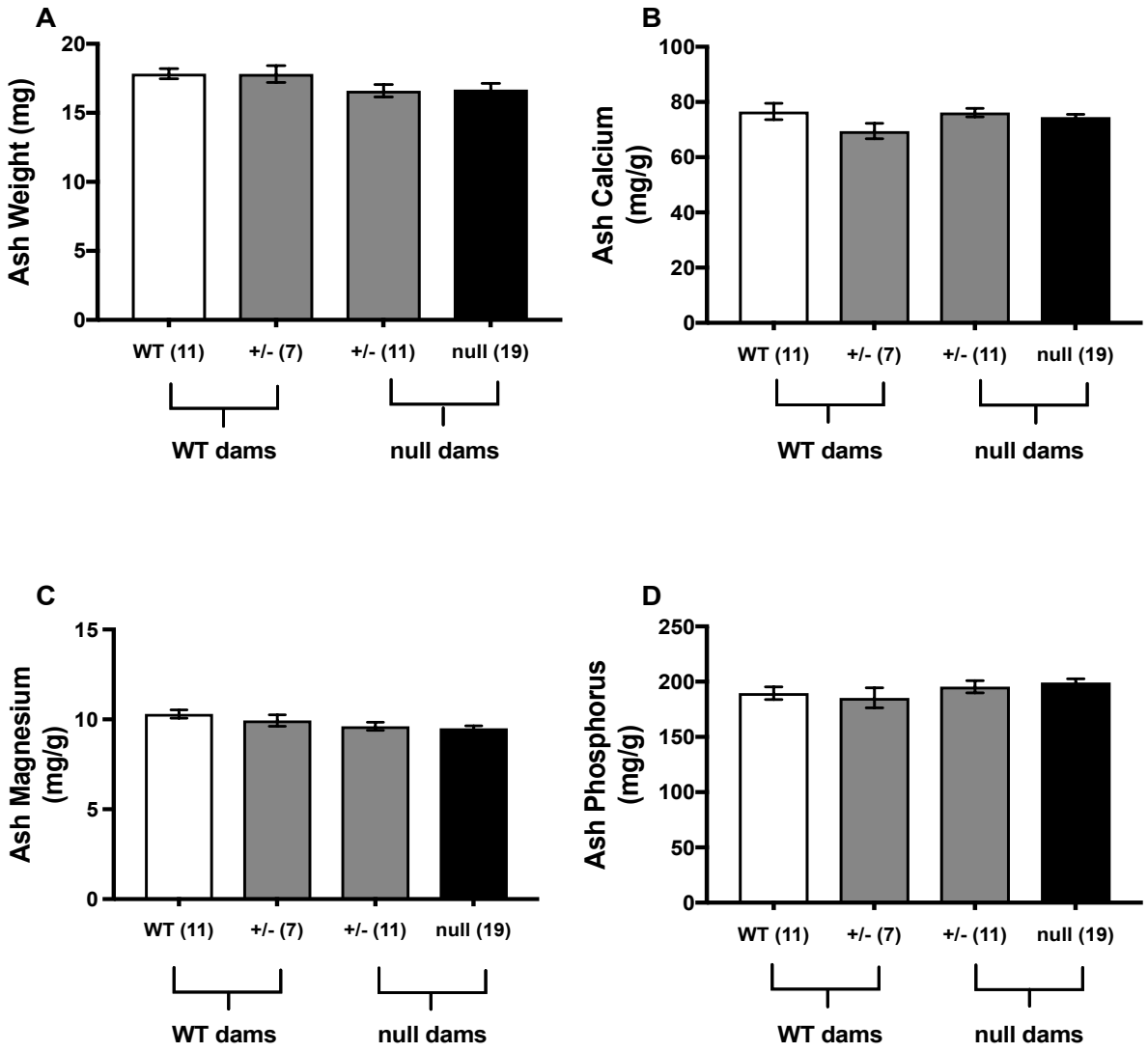

Figure 14. Fetal ash weight and skeletal mineral contents (ED 18.5).

The whole bodies of the *Cyp27b1* mutant fetuses born from *Cyp27b1* null and WT dams were reduced to ash to assess the (A) weight of the ash and the mineral contents of (B) calcium, (C) magnesium and (D) phosphorous. There were no between genotype-group differences in any of the parameters assessed herein. The number in parentheses represents the number of fetuses studied. Means reported ± SEM.

4 Discussion

The present study examined the role of calcitriol in fetal mineral and bone metabolism by studying the consequences of ablating the *Cyp27b1* gene, which encodes the 1α-hydroxylase enzyme that synthesizes calcitriol. Overall, *Cyp27b1* null fetuses born from *Cyp27b1*$^{+/-}$ dams exhibited normal serum and amniotic fluid calcium and phosphorus, serum PTH and FGF23, placental transport of calcium and phosphorus, and normally-developed skeletons with normal mineral content. However, the *Cyp27b1* null fetuses had low rather than undetectable serum calcitriol, likely due to transplacental transfer of calcitriol from the mother. Thus, in retrospect, this wasn't a study of true absence of calcitriol.

It is well known that calcitriol has a critical role in regulating mineral homeostasis and bone development in both neonates and adults (73). Low levels of calcitriol cause hypocalcemia, hypophosphatemia, hyperparathyroidism, and lead to rickets in children and osteomalacia in adults (72). To date, very few studies have examined the role of calcitriol in fetal mineral and bone metabolism (31,77,88,91,148,154-156). Previous work by others involving VDR ablation models have shown that *Vdr* null fetuses have normal serum minerals, PTH and skeletal morphology and mineralization (31,88). Human data also support that calcitriol may not play an essential role in regulating fetal mineral homeostasis (157). However, *Vdr* null fetuses exhibited a significant increase in placental calcium transport and serum calcitriol compared to their *Vdr*$^{+/-}$ and WT siblings (31). A similar result was found by a group in Belgium, where *Vdr* null females were mated to *Vdr*$^{+/-}$ males, which generated only *Vdr* null and *Vdr*$^{+/-}$ fetuses. The study showed that *Vdr* null fetuses had increased placental calcium transport compared to their *Vdr*$^{+/-}$ siblings (31,117). This increase in placental calcium transport in the absence of VDR is possibly

mediated through non-VDR pathways. Therefore, the present study aimed to investigate the role of calcitriol in fetal bone and mineral homeostasis in mice lacking calcitriol, the *Cyp27b1* knockout model (32).

My findings demonstrated normal fetal serum calcium, phosphorus, PTH, skeletal morphology, skeletal ash weight and ash mineral content in *Cyp27b1* null fetuses. These findings are in agreement with that of previous work by others using progenies of rodents with disrupted vitamin D physiology due to lack vitamin D, VDR or calcitriol, such as severely vitamin D-deficient rats (155,156), 1α-hydroxylase null pigs (88), and $Vdr^{+/-}$ and *Vdr* null mice (31,73). Taken together, these findings would imply that *Cyp27b1* nulls and *Vdr* nulls should be identical. Contrary to this notion, striking differences in their function and gene expression have been reported and are discussed in sections below. In addition to these differences, previous studies have also reported reduced serum FGF23 levels in *Vdr* null fetuses (117,158) in comparison to my findings showing normal serum FGF23 levels in *Cyp27b1* null fetuses.

Earlier studies using the offspring of vitamin D-deficient rats did not assess placental calcium transfer (155,156). Hence, we further investigated the rate of calcium and phosphorus crossing the placenta. Consistent with our serum, amniotic fluid and skeletal mineral content findings, *Cyp27b1* null fetuses of $Cyp27b1^{+/-}$ dams had similar rates of calcium and phosphorus placental transfer compared to their $Cyp27b1^{+/-}$ and WT littermates. This observation contradicts the notion that *Cyp27b1* nulls and *Vdr* nulls are identical. Previous studies in our laboratory and another group in Belgium showed that *Vdr* null mice had increased placental calcium transport (31,117) but had normal placental phosphorus transport (159). Therefore, high levels of calcitriol

act primarily to increase calcium transport only in the *Vdr* null. Importantly, the normal placental calcium and phosphorous transfer in *Cyp27b1* nulls, compared to the increased placental calcium transport in *Vdr* null fetuses (31), also suggest a possibility for calcitriol to regulate placental calcium transport through its actions on different receptors other than classic VDR. This may also explain why prior animal studies found that pharmacological treatment with calcitriol increased the rate of placental calcium transport (112-114,157).

Fetal kidneys and the placenta are well known to express *Cyp27b1*, which converts 25-hydroxyvitamin D_3 to calcitriol (160-162). The qRT-PCR analyses of the fetal placental and kidney tissues demonstrated an absence of the functional *Cyp27b1* mRNA in the *Cyp27b1* null fetal kidneys and reduced *Cyp27b1* mRNA in the placenta of null *Cyp27b1* mice relative to that of the corresponding *Cyp27b1* mRNA levels in tissues of WT mice. The presence of detectable levels of *Cyp27b1* mRNA in the placenta of null *Cyp27b1* fetuses is likely due to presence of maternal tissue. Furthermore, analyses of the expression of various calciotropic and phosphotropic genes in the placenta and fetal kidneys showed no significant changes in the expression levels of *Trpv6*, calbindin-D_{9k}, Ca^{2+}-ATPase *Napi2a*, *Napi2b*, *Napi2c*, *Vdr*, *Klotho* and *Fgf23* following *Cyp27b1* ablation. Normal placental expression of calbindin-D_{9k} and Ca^{2+}-ATPase and increased expression of *Trpv6* in placentas of vitamin D-deficient and/or *Vdr*-null rodents has been previously demonstrated (17,31,116,154). This finding further highlights the difference between the *Vdr* null and the *Cyp27b1* null fetuses, which have reduced expression of *Cyp24a1* in the fetal kidneys. As calcitriol is the main in vivo regulator of the expression of the CYP24a1 enzyme that catalyzes the synthesis of 24, 25$(OH)_2D_3$, the reduction of *Cyp24a1* mRNA is not surprising given the reduction in the levels of calcitriol. In contrast to the increased

expression of *Pthrp* in the placenta of *Vdr* null mice (31), my findings demonstrated a reduction in the expression of *Pthrp* mRNA in the placenta of *Cyp27b1* null fetuses. The low *Pthrp* expression in my studies may be attributed to the low levels of calcitriol and the high levels of *Pthrp* mRNA in *Vdr* nulls may be explained by the high levels of circulating calcitriol (31). Calcitriol typically circulates at <50% of the maternal value in fetal rodents (72). Consistent with this, serum levels of calcitriol in the *Cyp27b1* fetuses were ~50% less than the serum calcitriol levels in their *Cyp27b1*$^{+/-}$ dams. In *Cyp27b1* null fetuses, we expected calcitriol to be undetectable as previously reported in the adult null *Cyp27b1* mice (32). However, my findings showed reduced, but detectable, calcitriol levels in the serum of *Cyp27b1* null fetuses compared to their WT and *Cyp27b1*$^{+/-}$ littermates born from *Cyp27b1*$^{+/-}$ dams. There were also no differences in the fetal serum levels of other Vitamin D metabolites such as 24,25$(OH)_2D_3$ and 25OHD_3. It is likely that calcitriol found in the serum of *Cyp27b1* null fetuses was the result of placental transfer from their *Cyp27b1*$^{+/-}$ dams. However, prior studies in Hanover pigs (88) and rats (90) have suggested that calcitriol does not cross the placenta. In contrast, my notion of transplacental passage of calcitriol is supported by work involving placentas from human (97) and lambs (96), which demonstrated that radiolabeled calcitriol readily crosses the placenta. Thus, it is possible that there are inter-species differences in the ability of calcitriol to pass through the placenta, and this remains to be investigated systematically in mice. Moreover, since calcitriol and its precursor 25OHD differ by only one hydroxyl group, and since 25OHD readily crosses the placenta (157), it is likely that they both have this ability. Hence, to address the issue of transplacental transfer of calcitriol from mother to fetus, a preliminary study was conducted to assess fetal mineral homeostasis of *Cyp27b1* null mice born from *Cyp27b1*$^{-/-}$ mothers.

My findings showed no significant differences in the serum phosphorous levels and skeletal mineral content of *Cyp27b1* null fetuses born of *Cyp27b1* null dams, when compared to that of their *Cyp27b1*$^{+/-}$ littermates or the WT and *Cyp27b1*$^{+/-}$ fetuses of WT dams. Likewise, serum calcium levels of *Cyp27b1* null fetuses born of null dams were no different from their *Cyp27b1*$^{+/-}$ littermates, but both were significantly higher than those in the WT and *Cyp27b1*$^{+/-}$ fetuses of WT mothers. Taken together, it is plausible that calcitriol is not required for the maintenance of fetal mineral and bone homeostasis. However, the fact that whether these *Cyp27b1* null fetuses born of *Cyp27b1* null dams completely lacks calcitriol remains to be investigated. Due to time constraints, only serum levels of calcium and phosphorus, and skeletal ash and mineral contents were assessed in these mice.

Further investigation on the *Cyp27b1* null fetuses born of *Cyp27b1* null dams were undertaken by another MSc student, and together our findings were recently published (153). Specifically, *Cyp27b1* null fetuses born of *Cyp27b1* null dams were completely devoid of calcitriol, confirming that maternal transfer of calcitriol from *Cyp27b1*$^{+/-}$ dams to their *Cyp27b1* null fetuses, can provide calcitriol to fetuses that lack the ability to synthesize it (153). There were also no differences in serum calcium and phosphorus, PTH, FGF23, renal excretion of calcium and phosphorus into amniotic fluid, skeletal mineral content/morphology, placental calcium transport, and placental expression of most calciotropic and phosphotropic genes compared to their littermates (153).

Both of our findings, taken together, demonstrated conclusively that calcitriol does not play a role in fetal mineral homeostasis and skeletal development (153). These findings are in

agreement with all prior models of disrupted vitamin D physiology except that in *Vdr* null fetuses there is high circulating concentrations of calcitriol, increased placental calcium transport and increased placental expression of *Pthrp* and *Trpv6*. In contrast, *Cyp27b1* null fetuses born of *Cyp27b1* null dams display no detectable levels of calcitriol, normal placental transfer of calcium, and normal placental expression of *Pthrp* and *Trpv6*. These findings suggest that the increased levels of calcitriol in *Vdr* null fetuses (31) may act through a receptor or receptors other than classic VDR to stimulate placental calcium transport and gene expression. One candidate is protein disulfide isomerase family A, member 3 (Pdia3). Recent data suggested that this protein acts as a rapidly responsive plasma membrane receptor for calcitriol (163-167). Although the *Pdia3* mutation is embryonically lethal in mice, the heterozygotes have a skeletal phenotype similar to *Vdr* nulls (165,166). Our findings in the published paper showed that *Pdia3* was reduced in *Cyp27b1* null fetuses and increased in *Vdr* null fetuses implying that the high levels of calcitriol are possibly acting through this receptor (153). This hypothesis remains to be tested.

4.1 Limitations

As already noted, a significant limitation of the present study was that the *Cyp27b1* null fetuses were not devoid of calcitriol as expected when born from *Cyp27b1*$^{+/-}$ mothers. While further investigation of fetal serum calcitriol levels of *Cyp27b1* null fetuses born from null dams was necessary to establish the role of calcitriol in fetal mineral and bone homeostasis, time constraints precluded me completing these experiments.

Another limitation of the present study was that the ELISA kit used to measure the calcitriol levels was originally designed for human serum samples that require 500 µl of serum. However, as mentioned in the materials and methods, the minimum sample size required for the assay was 120 µl. The maximum amount we could get was about 35-40 µl of fetal serum. Thus, I pooled three or four samples together to get the amount needed for 1 sample. This further extended the completion time of the project and prevented me from fully completing the *Cyp27b1* null dam study. Furthermore, the mating strategy of WT and *Cyp27b1* null mothers in the *Cyp27b1* null dam study did not enable the WT and *Cyp27b1* null fetuses to be compared within the same litter, since *Cyp27b1* null dams had only *Cyp27b1*null and *Cyp27b1*$^{+/-}$ fetuses and WT dams had only WT and *Cyp27b1*$^{+/-}$ fetuses.

It is also important to remember that there is always a possibility for physiological compensation following inactivation of any gene. Thus, *Cyp27b1* gene inactivation may not necessarily equate to the absence of non-classical actions of calcitriol and does not prevent compensatory modulation in the expression of its related genes. Further studies involving the generation of mice with combined ablation of *Vdr* and inactivation of *Cyp27b1* genes may also prove useful in understanding the role of calcitriol in fetal mineral homeostasis and skeletal development.

4.2 Summary & conclusion

In summary, by comparing the WT, *Cyp27b1*$^{+/-}$ and *Cyp27b1* null fetuses born from *Cyp27b1*$^{+/-}$ mothers, our results indicate that the ablation of *Cyp27b1* does not affect fetal serum and amniotic fluid calcium and phosphorus levels, calcium and phosphorus placental transfers, and skeletal development and mineral deposition. However, my findings herein do not conclusively

rule out the possibility of calcitriol having a role in fetal mineral and bone homeostasis. This is because the *Cyp27b1* null fetuses born from *Cyp27b1*$^{+/-}$ dams were not devoid of calcitriol as expected. Therefore, while this study failed to investigate fetuses completely devoid of calcitriol, it confirmed that transplacental passage of calcitriol from the mother to the fetus does occur. Furthermore, this also suggests that *CYP27B1* null human neonates (vitamin D dependent rickets type 1 or VDDRI) will be born with maternal calcitriol in their circulation.

Whilst my preliminary findings for *Cyp27b1* null fetuses born from null mothers were included in my thesis, the studies on these mice were systematically investigated by another student, and our collective results were recently published (153). Taken together, our findings demonstrated conclusively that calcitriol does not play a role in fetal mineral homeostasis and skeletal development. Furthermore, my findings herein showing normal placental calcium transport in *Cyp27b1* null fetuses, compared to increased placental calcium transport in *Vdr* null fetuses (31), suggest that calcitriol regulates placental calcium transport by acting through receptors other than classical VDRs. It is also important to remember that there is always a possibility for physiological compensation following ablation of any gene. Thus, *Cyp27b1* gene ablation may not necessarily equate to the absence of non-classical actions of calcitriol and does not prevent compensatory modulation in the expression of its related genes. Further studies involving the generation of mice with combined ablation of *Vdr* and *Cyp27b1* genes may also prove useful in understanding the role of calcitriol in fetal mineral homeostasis and skeletal development.

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6 APPENDICES

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