Placental parathyroid hormone-related protein (PTHrP) regulates systemic mineral metabolism but not skeletal development in the fetus

By

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ABSTRACT

Global deletion of *Pthrp* results in lethal chondrodysplasia, hypocalcemia, hyperphosphatemia, and reduced placental calcium transport. Conditional deletion of *Pthrp* from chondrocytes and osteoblasts have shown that locally produced PTHrP acts as a paracrine factor to control skeletal development. However, the source and role of PTHrP in fetal circulation are uncertain. We set out to determine if circulating fetal PTHrP derives from the placenta and controls systemic mineral concentrations and placental calcium transport. We used established models, Tpbpa/Ada-*Cre* mice and floxed *Pthrp* mice, to create a putative trophoblast-specific deletion of *Pthrp* (Tpbpa/Ada-*Cre*; $Pthrp^{fl/fl}$). We mated Cre-$Pthrp^{fl/+}$ females to $Pthrp^{fl/fl}$ males. Expected fetal genotypes are $Pthrp^{fl/fl}$ and $Pthrp^{fl/+}$, with half of each expressing Cre. The four fetal genotypes appeared 1:1:1:1, indicating no prenatal losses. Cre-$Pthrp^{fl/fl}$ fetuses showed low serum calcium and higher serum phosphorus compared to their $Pthrp^{fl/fl}$ littermates and maternal values. Weights of placentas, intact fetuses, and fetal skeletal ash did not differ between the genotypes, nor did the fetal skeletal ash content of calcium, phosphorus, and magnesium. 5-micron longitudinal sections of fetal tibiae showed no change in length, morphology, or mineralization pattern by von Kossa staining. qPCR revealed increased placental expression of the gene for calbindin-D-9k (*S100g*), a gene involved in placental calcium transport. These findings suggest that placental PTHrP regulates systemic mineral levels in fetuses but has no direct role in skeletal development.

GENERAL SUMMARY

PTHrP is an important factor during fetal bone development. Mice with globally deleted PTHrP die shortly after birth and display impaired bone development, low serum calcium, higher serum phosphorus, and reduced transport of calcium across the placenta. We set out to investigate the role of PTHrP, from the placenta, in fetal bone and mineral homeostasis. To do this, we selectively deleted PTHrP from the placental trophoblast cells. The results showed low serum calcium and high serum phosphorus in the fetal mice that lacked placental PTHrP, but no significant change in weights of placentas, intact fetuses, or fetal skeletal ash. There was also no change in fetal skeletal lengths, morphology, or mineralization pattern, and no change in ash mineral content of the fetuses. There was an increase in placental expression of *S100g*, a gene that encodes a calcium transporter. The results suggest that placental PTHrP regulates systemic mineral levels in fetuses but has no direct role in skeletal development.

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1α-Hydroxylase……CYP27B1

24-Hydroxylase(24OHase)……...CYP24A1

25-Hydroxyvitamin D……...25OHD

25-Hydroxylase……...CYP21R

Analysis of variance…….. ANOVA

Blomstrand lethal chondrodysplasia……BLC

Bone Multicellular Units……..BMU

Canadian Institue of Health Research……CIHR

Casearean section……...……C-section

Calcitriol (1,25-dihydroxyvitamin D)……..……. $1,25(OH)_2D$

Calcium-sensing receptor…… CaSR

Complementary DNA……..cDNA

Cyclic adenosine monophosphate……...cAMP

Deionized water……....dH_2O

Embryonic day……ED

Extracellular fluid……ECF

Fibroblast growth factor-23…….. FGF-23

FGF tyrosine kinase receptor 1……..FGFR1

Humoral hypercalcemia of malignancy……HHM

Hydroxyapatite crystals……..... $Ca_{10}(PO_4)_6(OH)_2$

Immunohistochemistry…….……..…IHC

Indian hedgehog……. Ihh

In situ hybridization……ISH

Interleukin 6……..IL-6

Jansen's metaphyseal chondrodysplasia……JMC

Millimolar…….….. mM

Osteoprotegerin……. OPG

Parathyroid hormone……..PTH

Parathyroid hormone related protein……..PTHrP

Parathyroid hormone receptor type 1……....PTH1R

Polymerase chain reaction……..PCR

RANK ligand…….. RANKL

Receptor activator of nuclear factor-κB…….……RANK

Retinoid X receptor……RXR

Standard deviation……...…….SD

Threshold cycle……. C_T

Transient receptor potential cation channel subfamily V member 6…….TRPV6

Type 2a sodium-phosphate cotransporters…….NPT2a

Type 2c sodium-phosphate cotransporters…….NPT2c

Vitamin D binding protein…….DBP

Vitamin D receptor……VDR

LIST OF APPENDICES

I. INTRODUCTION

1.0 Preamble

The following thesis involves the study of fetal mice that have been engineered to lack expression of parathyroid hormone related protein (PTHrP) in trophoblast cells of the placenta. To understand the context of the data provided, I will begin with a brief background on adult bone and mineral homeostasis, fetal bone and mineral homeostasis, and current research from studies involving human and animal models on PTHrP. Following this, I will cover the rationale, hypothesis, purpose, and objectives of this research project.

Serum phosphorus exists predominately as inorganic phosphates (dihydrogen and monohydrogen phosphate). In bone, phosphorus is mostly in the form of hydroxyapatite and in soft tissues and extracellular fluid (ECF) it mainly exists as organic phosphates complexed with proteins, carbohydrates, and lipids. For simplicity and consistency, and because phosphorus and not phosphate is what is reported from serum and urine assays, the term phosphorus will be used to reference all its forms throughout this thesis.

As there will be many references to genes and proteins, the following international standard nomenclature will be used. Murine names begin with an initial capital followed by lowercase, with genes italicized and proteins in normal font. Human names are entirely in upper-case with genes italicized and proteins in normal font. When both animal and human models are being referenced, the nomenclature will follow the human nomenclature; if both the gene and the protein are implied, then the protein form will be used.

1.1 Adult Bone and Mineral Homeostasis

1.1.1 Bone Structure and Function

Bone is a dynamic tissue which has many important roles in the body. The skeleton supports the bodies of all vertebrate organisms, including mammals, and provides protection for their internal organs.[1-3] It allows for locomotion, as muscles, tendons, and ligaments attach to bone, and hosts hematopoiesis within bone marrow. Bone is also the major source of minerals (calcium, phosphorus, and magnesium) in the body as it stores the majority in an accessible form within its structure.[1-3] There are two main types of osseous tissue that make up bone. The first is cortical bone, also known as compact bone, and the second is trabecular bone, also known as cancellous bone (Figure 1).[1-3] Cortical bone makes the majority of the skeleton and can be recognized to form the outer shafts of long bones.[1-4] Its very dense structure with low porosity (5-10%) contributes much of the strength of the skeleton. Trabecular bone has a more mesh-like structure with a high porosity (75-95%) and is found at the ends of long bones, in flat bones, and makes up much of the vertebral bodies (Figure 1).[1-4] When examining cortical bone further it is evident that bone has two major types of tissues, lamellar and woven bone.[4] Lamellar bone forms slowly and is highly organized lamellae, parallel layers, comprising of collagens fibers and mineral crystals.[4] Lamellae create circumferential bands of bones, which look like tree rings. Woven bone is quicker formed and poorly organized tissue that has collagen fibers and minerals crystals randomly arranged.[4] While woven bone can form much quicker than lamellar bone it is weaker. Woven bone can become more mineralized compared to lamellar bone.[4] Osteon, also known as Haversian system, is the functional unit of cortical bone. A

singular Haversian system is made up of lamella and canaliculi around a circular canal, known as the Haversian canal, that contains blood vessels and lymphatic vessel.[4]

Bone is often viewed as a static, inert structure. However, it is an extremely dynamic organ, that is ever-changing.[1-3] There are three processes which account for the formation and reformation of bone from fetal development to adulthood. These processes are growth, modelling, and remodelling.[4] Growth allows for the initial formation and elongation of bone through ossification during fetal development and childhood, it will be discussed in greater detail in section 1.2.3 as this process mainly occurs before puberty/adulthood.[4] Modelling is the process of osteoblasts forming new bone on surfaces that have not been previously excavated by osteoclasts. Remodelling is the sequential process by which osteoclasts breakdown old bone and osteoblasts follow closely behind in their and lay down new bone at the same site.[1-4]

Osteoclasts, osteoblasts, and osteocytes are the cells responsible for this dynamic nature of bone.[2,4] The process by which old bone is remodelled is a complicated cycle comprised of three key steps. Osteocytes play a key role by sensing areas of damage (such as micro-cracks) or increased stress in bone, and direct osteoclasts to those areas. Once these areas are detected, the first key step is the breakdown and resorption of the old bone by the osteoclasts. This is followed by the second transitional step from resorption to formation. And finally, the third key step is the formation of new bone by the osteoblasts. This normally results in the same amount of bone as was there previously except that it is now stronger without microdamage.[2,4] This cycle is important, in adults, to maintain a normal, healthy skeleton, as well as for mineral homeostasis.[1,2,4] Osteoclasts and osteoblasts that work together at specific sites of bone

remodelling by forming a coordinated assembly of cells are known as the Bone Multicellular Units (BMUs).[4] The BMUs are made up of around 10 osteoclasts and several hundred osteoblasts and go through three principle stages: activation (A), resorption (R), and formation (F) known and the A-R-F sequence.[4] Bone remodelling occurs at the surface of trabecular bone, however, osteoclasts are also able to invade into the cortical bone, allowing bone remodelling to happen both on the surface and inside cortical bone.[4] This allows old cortical bone to be reabsorbed and new bone to be built in its place. Because of its greater surface due to its mesh-like structure, trabecular bone is broken down faster and is more susceptible to damage when net bone loss occurs.[1]

Bone also serves as a reservoir for the inorganic ions: calcium, phosphorus, and magnesium. It allows these ions to be quickly mobilized based on the body's needs, in order to maintain mineral homeostasis.[1] During the bone remodelling process these minerals are either released into the circulation or taken up and stored by the bone as hydroxyapatite crystals [$Ca_{10}(PO_4)_6(OH)_2$].[1] The need for minerals in the body is tightly regulated by local and systemic factors that work together by sending signals in a feedback and feed-forward fashion, depending on the body's requirements.

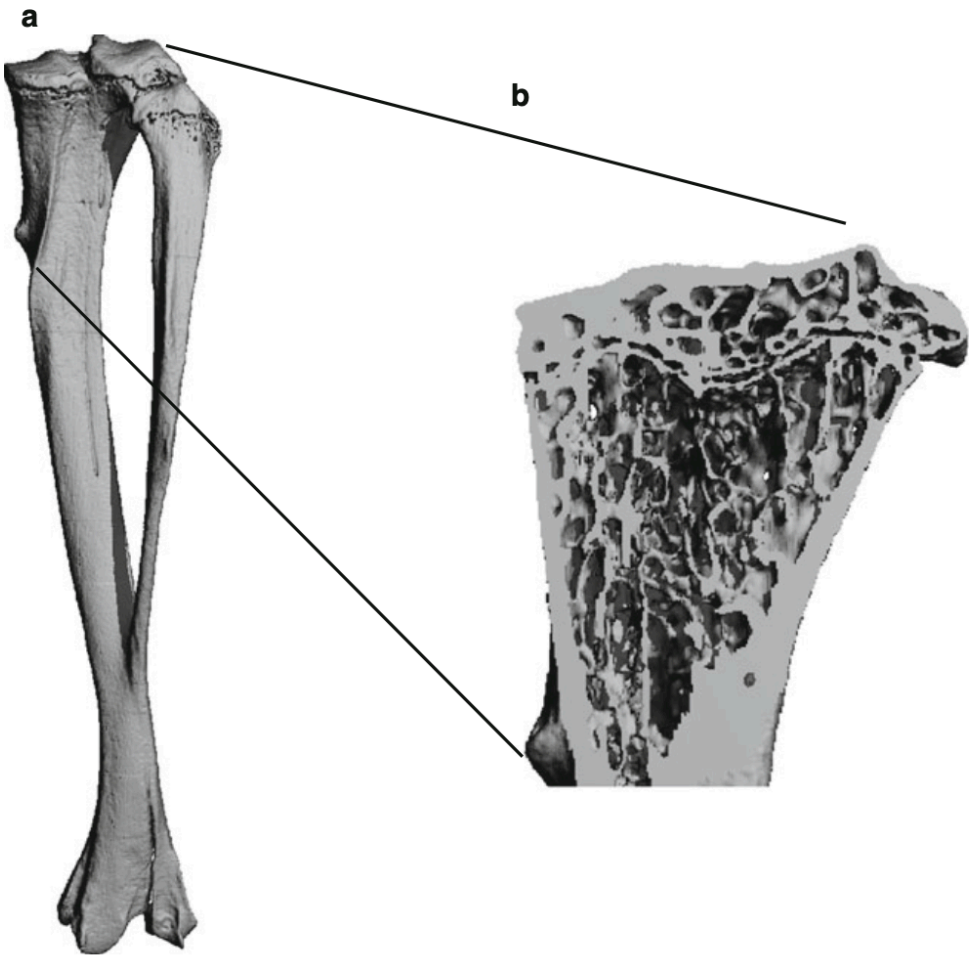

Figure 1**: Cortical and trabecular bone structure in the tibia.**[1] Whole tibial structure obtained by micro-computed tomography, 12 microns isotropic spatial resolution.[1] (a) Displays a tibia and the dense cortical bone that surrounds it. (b) The solid outside is the dense cortical bone that surrounds the mesh-like trabecular bone. Because of this mesh-like surface, the trabecular bone has a greater surface area and is more susceptible to osteoclast activity. Used with permission from John Wiley and Sons © 2018 (Appendix A).

1.1.1.1 The Bone Cells

1.1.1.1.1 Osteoclasts

Osteoclasts are large multinucleated cells that derive from hematopoietic progenitor cells which makes them a member of the monocyte-macrophage family.[3,5] They are responsible for the breakdown of old bone, through their secretion of acids and digestive enzymes. Osteoclasts also allow minerals to be released from the hydroxyapatite into the blood for their circulation and use in other biological pathways throughout the body.[4] Osteoclast activity is crucial for mineral homeostasis and is tightly regulated by several factors including hormones and osteoblasts. Parathyroid hormone (PTH) and the receptor activator of nuclear factor-κB (RANK)/ RANK ligand (RANKL) and osteoprotegerin (OPG) signalling pathway are key regulators in osteoclast activity. PTH is released from the parathyroid glands when serum calcium levels are low. PTH increases the activity of osteoclasts indirectly by stimulating the osteoblasts to release RANKL and promotes the differentiation, action, and survival of the osteoclasts.[1,6,7] Osteoclasts will then glide along the surface of the bone secreting acids and digestive enzymes, that cause the degradation of bone, releasing collagen and mineral. The calcium that is released goes back into circulation and causes the calcium-sensing receptor (CaSR) on the parathyroid to inhibit the secretion of PTH.

1.1.1.1.2 Osteoblasts

Osteoblasts are the primary cells responsible for bone formation and reformation, through a process called osteogenesis. Osteoblasts derive from mesenchymal stem cells. These stem cells have the potential to proliferate and differentiate into other connective tissue cell types such as chondrocytes and adipocytes, the cells that respectively form cartilage and adipose tissue.[3,8] A

stimulus causes the mesenchymal stem cells to differentiate into osteoprogenitor cells, these cells will later differentiate into osteoblasts.[8] As the osteoclasts degrade bone, osteoblasts follow behind on the surface of the bone and secrete type I collagen and proteoglycans. This forms the organic component of the bone matrix, also known as osteoid. They also secrete minerals, the inorganic component.[3,8] The minerals form a crystal complex, the hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] which allows minerals to be stored in bone and contribute to its strength.[1]

Osteoblasts play a role in the mineralization of the osteoid through the liberation of matrix vesicles and the deposition of calcium and phosphorus. The deposition of the bone organic matrix allows for an increase in bone density and strength. This is essential in the initial building of the skeleton during fetal development.[8] While osteoblasts and osteoclasts work individually to form and break down bone respectively, they do coordinate the bone remodelling process through paracrine signalling events (RANK/RANKL/OPG pathway).[8] An imbalance in the relationship between osteoclasts and osteoblasts, such as in response to estradiol deficiency or inadequate dietary calcium intake, can lead to osteoclasts degrading more bone than osteoblasts can form. This will cause a decrease in bone density and can lead to bone disorders, such as osteoporosis.

1.1.1.1.3 Osteocytes and Bone Lining Cells

Osteoblasts can also become osteocytes and bone lining cells.[4] Osteocytes are osteoblasts that have become embedded within the bone matrix they have produced.[3] Originally it was thought that these cells did not have much of a role in bone remodelling. It is evident, however, that they comprise a living network that

communicates with themselves and the other bone cells in response to loading, unloading, and damage to the skeleton. They are able to form bone, like osteoblasts, and resorb bone, like osteoclasts. Osteocytes can also act like osteoblasts to reform bone that was broken down.[4] They will express the same genes and proteins that osteoblasts do, and secrete the same organic matrix that gets mineralized, creating stronger, new bone.[4] If osteocytes undergo apoptosis, then the affected area of bone is less able to respond to and repair damage, such as from micro-cracks or osteoporosis.[4] They can act like osteoclasts in a process known as osteocytic osteolysis. They will secrete the same acids and proteins as the osteoclasts to break down bone and release stored mineral. This is important for the quick acquisition of mineral from bone into circulation as the combined surface area of the osteocytes is approximately 10 times that of the surface area of the trabecular bone in humans.[9] They occupy a space known as the lacunae in the osteon, the functional unit of the cortical bone. They are said to be involved in cell signalling and the maintenance of the bone matrix.[3] Osteocytes are connected to each other and to cells on the bone surface through arm-like projections known as canaliculi, allowing them to send signals through the lacuna-canalicular network that influence bone remodelling.[10] Much is still not understood about these cells however it is clear that they are important for the bone remodelling system. Similar to osteocytes, bone lining cells were osteoblasts that have remained on the surface of bone once bone formation has ceased.[4] After the production of the new bone matrix, the bone lining cells flattened on the surface of the bone and become quiescent. These cells communicate with the osteocytes and are thought to be responsible for the transfer of mineral in and out of bone.[4]

1.1.1.2 Bone Remodelling through RANK/RANKL/OPG signalling

As previously described, bone remodelling is the process in which osteoclasts degrade old bone and osteoblasts build new bone.[6] This process is critical for adult bone homeostasis and involves balanced coordination in bone formation and resorption to support bone mass and systemic mineral homeostasis.[6] One of the major signaling pathways that controls osteoclast and osteoblast activity is the RANK/ RANKL and OPG signalling pathway.[6,7]The RANK/RANKL/OPG signalling pathway (Figure 2) regulates osteoclast formation, activation, and survival in normal bone remodelling in adults.[6,7] RANK, the receptor for RANKL, is expressed on the surface of pre-cursor and mature osteoclasts. When RANKL binds to RANK on the pre-cursor osteoclasts, they will begin to fuse together to form mature multinucleated osteoclasts, which are responsible for the breakdown of bone. RANK will continue to bind to these mature osteoclasts as they degrade the old bone.[6,7] OPG is another protein secreted from osteoblasts and it acts as a natural inhibitor of RANKL by binding to it and preventing its association to RANK on the osteoclast surface, thereby fulfilling a crucial role in the regulation of bone resorption.[6,7]

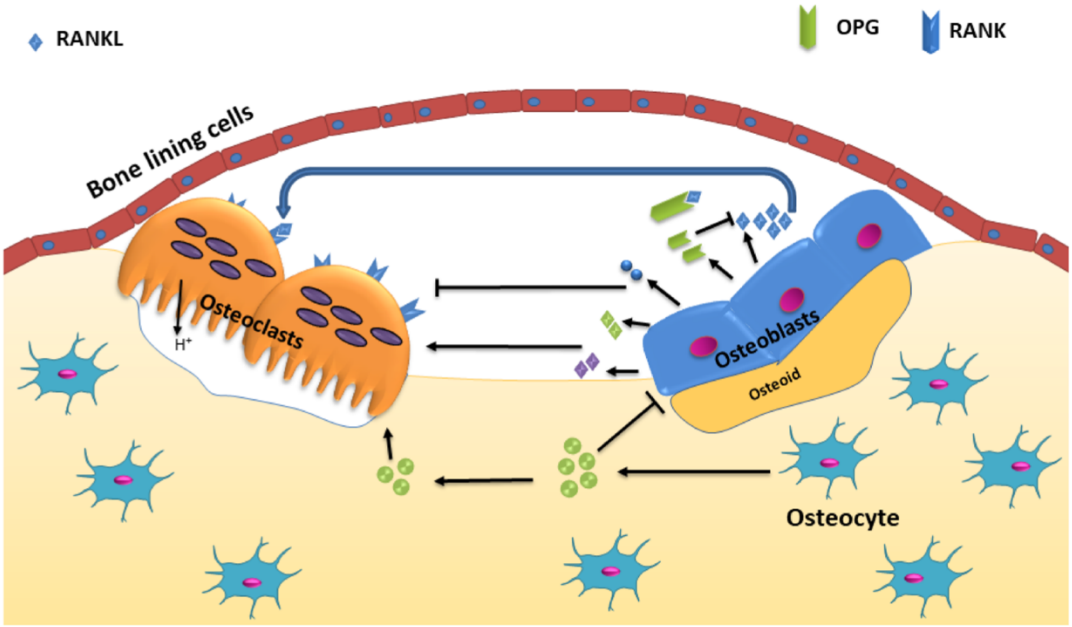

Figure 2: Bone Remodelling.[6] The osteoclasts, osteoblasts, and osteocytes are responsible for the bone remodelling process. Osteoclasts breakdown old bone and osteoblasts reform new bone. Osteocytes, trapped in the bone matrix, response to stimuli and communicate to adjacent cells. RANK/RANKL/OPG signalling pathway is a local factor regulating the activity of these cells. Figure adapted and used with permission from John Wiley and Sons © 2018 (Appendix A).

1.1.2 Serum Minerals

1.1.2.1 Calcium

Calcium is the most abundant mineral in the human body, with the adult body containing around 1 kg of calcium.[11] Approximately 99% of this calcium is stored in the bone in a complex with phosphorus (hydroxyapatite crystal) where it plays a significant role in the mechanical weight bearing properties of the skeleton.[11] The crystal also acts as a ready source of calcium to support biological systems and regulate blood ionized calcium levels (normal levels around 1.55 mmol/L and 1.32 mmol/L).[12] The 1% of calcium not stored in the bone is found in the blood, other extracellular fluids, and cells.[11]

In adults and children, the skeleton, intestines (largely the duodenum and the upper jejunum), and kidneys are key players in calcium homeostasis.[11,13] The average adult ingests approximately 1g of elemental calcium per day. Of that 1g, about 200 mg will be absorbed and 800 mg excreted.[11] From the 1 kg stored in the bone, approximately 500 mg is released through resorption and 500 mg is also deposited during bone formation when bone turnover is in balance each day. In the kidneys, 10 g of calcium is filtered each day but only about 200 mg or less gets excreted while the remainder is reabsorbed into the circulation.[11] If the amount of calcium being reabsorbed is out of balance with the amount of calcium being deposited it could contribute to hypocalcemia (low serum calcium) or hypercalcemia (high serum calcium).

1.1.2.2 Phosphorus

Phosphorus is another key mineral for skeletal integrity and is important for intracellular processes.[14] Phosphorus is found in the soft tissues of the body and as a component in organic

molecules. Approximately 80% of phosphorus in the body is stored as hydroxyapatite in the bone matrix.[14] As with calcium, bone is the primary reservoir for phosphorus that can be readily mobilized to regulate the levels of phosphorus in the blood. In adults and children, the skeleton, the intestines, and the kidneys play a vital role in the regulation of serum phosphorus concentrations.[13,15] Fibroblast growth factor-23 (FGF23), PTH, and calcitriol are key hormones responsible for phosphorus homeostasis in adults and children.

1.1.2.3 Magnesium

Magnesium is another key mineral stored in the bone. Approximately 99% of the total body magnesium is intracellular, with the remaining 1% in the ECF. Around 90% of the total body magnesium is found in the bone, muscle, and soft tissues with 0.3% found in the serum, of which a third of this is protein-bound.[16]

1.1.3 Calciotropic and Phosphotropic Hormones

The calciotropic and phosphotropic hormones include PTH, calcitriol, FGF-23, estradiol, and testosterone.[13,17] These hormones not only contribute to the regulation of calcium and phosphorus, but they also influence each other through feedback and feed-forward loops. The loss of any of these hormones can cause adverse effects in children and adults, leading to health problems and disease (for example bone disorders such as rickets or osteomalacia).[13,17]

1.1.3.1 PTH

PTH is one of the dominant regulators of calcium and bone metabolism in adults and other mammals.[18] It is released from chief cells in the parathyroid gland, and it is responsible for the

tight regulation of blood ionized calcium, between 1.1 to 1.3 millimolar(mM).[19] This level is the normal level that is required for bone mineralization and other important physiological pathways.[19,20] PTH secretion is controlled by several factors including blood ionized calcium, serum phosphorus, calcitriol, and FGF23. The relationship between blood ionized calcium and PTH secretion is a steep sigmoidal one, allowing significant changes in PTH secretion in response to very small changes in blood ionized calcium.[16] The ionized calcium concentration is detected at the surface of the parathyroid cells through CaSR to regulate the secretion of PTH.[19,20] CaSR senses the ambient ionized calcium concentration and inhibits PTH synthesis and release when calcium binds to it; conversely, it disinhibits the synthesis and release of PTH when less calcium is available to bind to the receptor. The CaSR is also expressed in kidney tubules where it inhibits calcium reabsorption and thereby leads to renal calcium excretion.[20]

PTH binds to and activates the Parathyroid Hormone Receptor Type 1(PTH1R), expressed by bone, kidneys, and intestines. The activation of the receptor triggers several intracellular signalling pathways.[19] In response to low calcium, the secretion of PTH directly increases renal reabsorption of calcium, stimulates the release of calcium from the bone by activating osteoclasts and osteocytes, and increases intestinal calcium absorption indirectly by increasing renal synthesis of calcitriol.[20] One of the main impacts PTH has on the release of calcium from bone is through its stimulation of the osteoclasts. PTH stimulates the production of interleukin-6 (IL-6) and RANKL by the osteoblasts. As explained previously, release of RANKL promotes bone resorption and consequently the release of calcium into the serum.[19,21]

1.1.3.2 Calcitriol

Calcitriol, the active form of vitamin D_3, is essential for regulating mineral metabolism in children and adults through its endocrine action on the homeostasis of calcium and phosphorus across three main tissues: the intestines, kidneys, and bone.[22,23] Vitamin D is converted into its active form in multiple steps (Figure 3). Vitamin D is either synthesized in the skin after UV exposure or ingested in the diet from food or supplementation, as either vitamin D_3 or vitamin D_2, and enters the bloodstream.[22,23] Once in the bloodstream it circulates bound to vitamin D binding protein (DBP) and will be circulated to the liver where it is first converted to 25-hydroxyvitamin D (25OHD) by 25-hydroxylase (CYP21R). Then 25OHD, bound to DBP, is circulated to the kidney where it is converted to calcitriol [$1,25(OH)_2D$] by 1α-hydroxylase (CYP27B1).[22,23] Once it has been converted into its active form, calcitriol will act upon the vitamin D receptor (VDR).

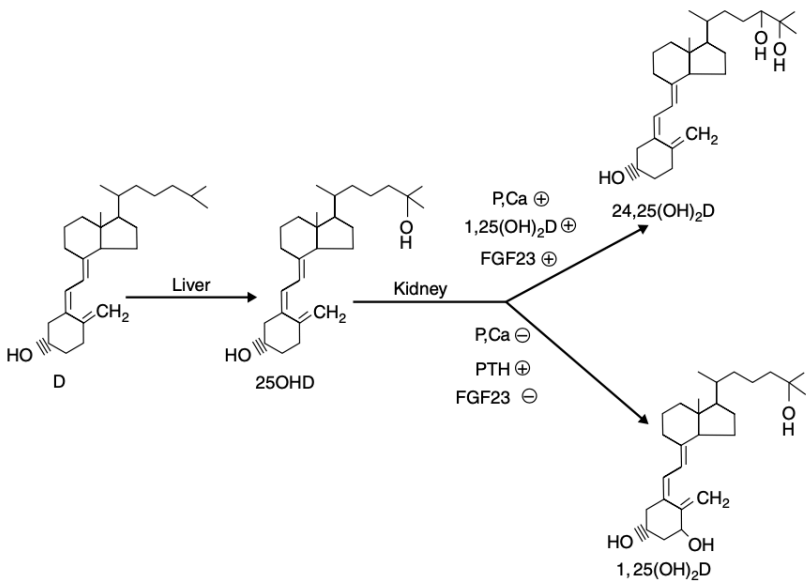

Figure 3: The metabolism of vitamin D into its active form, calcitriol.[22] Once it reaches the liver, vitamin D is converted to 25OHD by CYP21R. Then 25OHD is circulated to the kidney where it is converted to calcitriol [1,25$(OH)_2$D] by CYP27B1. 24- Hydroxylase (CYP24A1) catabolizes both 25OHD and calcitriol into inactive metabolites. Used with permission from John Wiley and Sons © 2018(Appendix B).

The VDR is a member of the steroid receptor family and is currently the only known receptor for calcitriol.[23] Calcitriol binds to VDR inducing a conformational change, then forms a heterodimer with retinoid X receptor (RXR) and coregulatory complexes, allowing binding to specific DNA. This results in the expression or transrepression of target genes.[23] One of the primary functions of calcitriol in adults and children is the maintenance of calcium homeostasis by the upregulation of intestinal calcium absorption and the upregulation of distal tubular calcium reabsorption by the kidney. The increase of calcium absorption in the gut and reabsorption in the kidney, facilitate skeletal mineralization by providing the needed mineral to osteoblasts. This increase in serum calcium in turn activates the CaSR in the parathyroids to inhibit the release of PTH.[23] Calcitriol also stimulates active phosphorus absorption in the intestines, however, the mechanism is still unknown. Calcitriol indirectly enhances phosphorus reabsorption in the kidney by lowering the levels of PTH.[23]

Calcitriol works in a classic endocrine fashion alongside PTH and FGF23 in a series of feed-forward and feedback loops.[22] PTH stimulates the production of calcitriol in the kidneys. Calcitriol activated VDR suppresses PTH production through both direct and indirect pathways. It will directly suppress PTH through transcriptional mechanism and indirectly by increasing serum calcium levels, causing the CaSR to suppress PTH secretion.[22] FGF23 has an opposing effect as it inhibits Cyp27b1 to reduce the production of calcitriol in the kidneys, as well as increasing the catabolism of calcitriol by increasing the expression of Cyp24a1. In bone, calcitriol will stimulate FGF23 production and upregulate klotho-α (discussed further in section 1.1.3.3).[22]

1.1.3.3 FGF23

Phosphorus is regulated by the phosphotropic hormone FGF23. The level of phosphorus in the serum is regulated by intestinal phosphorus absorption, renal phosphorus handling, and the dynamic equilibrium between extracellular phosphorus, phosphorus in the bone, and intracellular spaces.[24] In children and adults, FGF23 is mainly expressed in bone, especially the osteoblasts and the osteocytes, but the majority of its impact is seen in the kidneys, where FGF23 promotes renal phosphorus excretion.[24] The main determinant of serum phosphorus levels is believed to be the kidneys because approximately 80-90% of the phosphorus that is filtered from the glomeruli is reabsorbed in the proximal tubules.[24]

FGF23 binds to the FGF receptor-Klotho complex, specifically the receptor FGF tyrosine kinase receptor 1 (FGFR1) and the FGF23-specific co-receptor Klotho.[25] Klotho is a protein expressed in limited tissues, including the kidneys and parathyroid glands. It is believed that the tissue-limited expression of Klotho is what determines the target organs for FGF23.[24] The secretion of FGF23, from the osteoblasts or osteocytes, reduces the concentration of phosphorus in the serum by supressing the expression of type 2a and 2c sodium-phosphate cotransporters, NPT2a and NPT2c respectively, in the renal proximal tubules of the kidneys.[24] Increased concentrations of FGF23 inhibits Cyp27b1 and stimulates Cyp24a1. Both mechanisms work to decrease levels of calcitriol by reducing the synthesis and increasing the catabolism of calcitriol respectively, in turn reducing intestinal phosphorus absorption.[24-26] Figure 4 shows how PTH, calcitriol, and FGF23 work in a series of feed-forward and feedback loops to influence adult mineral homeostasis.

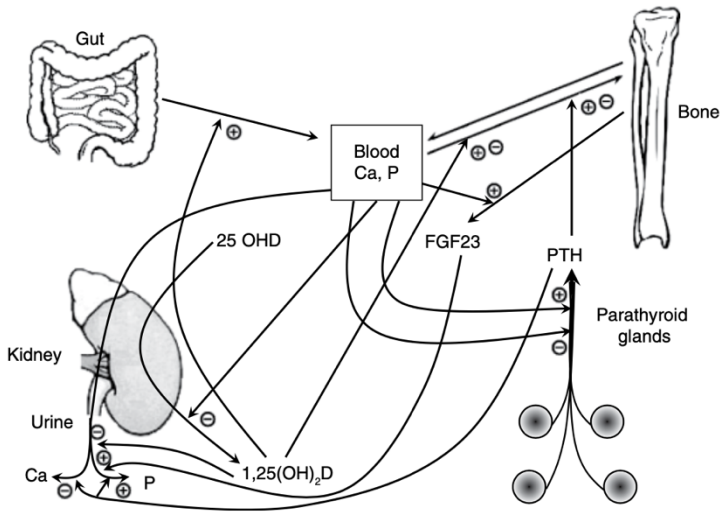

Figure 4: Adult bone mineral homeostasis, feedback loops.[22] PTH, calcitriol, and FGF23 work together in a series of feed-forward and feedback loops to regulate calcium and phosphorus homeostasis. Used with permission from John Wiley and Sons © 2018 (Appendix B).

1.1.3.4 Sex steroids

The length of bones and the robusticity of the skeleton are greatly influenced by sex hormones. During adolescence, after puberty, the male skeleton characterized by long bones with larger diameters and greater cortical thickness as compared to the female skeleton.[27] Estradiol, one of the main sex steroids, is responsible for the closure of the growth plates and long bone growth in both sexes during puberty. Because the female skeleton is exposed to higher levels of estradiol, from its production by the ovaries, the long bone growth ceases earlier as estradiol causes the epiphyseal closure through the acceleration of the loss of progenitor cells in the resting zone of the long bone growth plates.[28] The testes also produce estradiol but the prepubertal female produces eight times higher estradiol levels compared to males. Therefore, females go through an earlier growth spurt and earlier growth arrest in males.[28] Estradiol also influences the pelvic region and causes the female pelvis to expand more than the male pelvis.[28] The role of androgens, specifically testosterone, is also crucial for the male skeleton during puberty.[27] Androgens will be converted to estradiol by aromatase that is expressed in the growth plates.[28]

Estradiol deficiency is one of the major pathogenic factors associated with bone loss during menopause and leads to postmenopausal osteoporosis.[27] Estradiol also suppresses the release of RANKL and increases the secretion of OPG. Low levels of estradiol cause an increase in RANKL and a decrease in OPG, causing an increased osteoclast activity. This is the main influence during menopause that can lead to osteoporosis.[27] In men, testosterone deficiency leads

to estradiol deficiency that in turn contributes to bone loss through the RANK/RANKL/OPG pathway.[29]

1.1.4 Role of the skeleton

As previously described, bone regulates adult mineral homeostasis as it stores the majority of mineral, acting as a reservoir where these ions can be quickly mobilized into circulation. Bone remodelling is occurring continuously in healthy bone through the actions of osteoclasts, osteoblasts, and osteocytes. Bone remodelling is tightly regulated by local and systemic factors. As discussed, one of the local factors is RANKL/RANK/OPG signalling. There are other local factors but for simplicity, this is the only one discussed in this thesis.[6,7] The systemic factors include PTH, calcitriol, FGF23, and the sex steroids. All these factors tightly maintain bone and mineral homeostasis through multiple pathways and feedback loops that detect even the slightest changes in mineral concentrations.

1.1.5 Role of the kidneys

The kidneys are another organ responsible for mineral homeostasis in adults and children. The main renal regulation of calcium, phosphorus, and magnesium occurs through the glomerular filtration and tubular reabsorption or secretion.[30] The proximal renal tubular epithelial cell is the richest source of CYP27B1, the enzyme that converts 25OHD to calcitriol, and is therefore responsible for generating the amounts of calcitriol that are required for mineral homeostasis.[22] Endocrine regulation of CYP27B1 is mainly controlled at the level of transcription through the circulating PTH, the major stimulator of the production of calcitriol, and the circulating FGF23, the major inhibitor of calcitriol.[22] PTH stimulates the production of calcitriol by stimulating

CYP27B1 and inhibiting CYP24A1. As noted earlier, PTH and FGF23 have opposing effects to raise and lower calcitriol, respectively, through their actions on CYP27B1 and CYP24A1.[31] PTH and FGF23 have similar actions in the kidney tubules to inhibit renal phosphorus reabsorption and promote its excretion. PTH also acts to stimulate the reabsorption of calcium. The CaSR plays a role in calcium reabsorption too, as mentioned earlier.

1.1.6 Role of the intestines

The intestines are uniquely responsible for the acquisition of new minerals into the body. After dietary intake, the intestine, specifically the duodenum and the jejunum, replenish the mineral stores in the bone through the absorption of the new mineral.[13] The intestines are also responsible for the uptake of minerals in circulation through the body. Calcium and phosphorus are almost exclusively absorbed in the small intestine, mainly in the duodenum and the upper jejunum. However, passive transport of calcium can occur through the entire intestine.[13] Initially after birth, the passive absorption of calcium is facilitated by lactose but later switches to calcitriol-dependent active transport. However, in cases such as a high calcium diet, substantial calcium can passively cross the intestine.[13] Passive and active absorption of phosphorus is facilitated by FGF23 and can also occur throughout the entire intestine.[13]

1.2 Fetal Bone Development and Mineral Homeostasis

Bone and mineral homeostasis in adults and children can almost fully be explained through the interaction of several regulatory hormones (PTH, calcitriol, FGF23, and sex steroids) and certain organs (the skeleton, kidneys, and intestines).[13] However, with the exception of the skeleton itself, these hormones and organs play little to no role in fetal mineral homeostasis or fetal bone

development.[13] There are limited human data that directly examine fetal mineral homeostasis and fetal bone development due to ethical issues. . The human data that have been collected is mainly from cord blood samples taken from normal fetuses, abnormal preterm fetuses, and term fetuses, and from pathological examination of embryos and fetuses that died due to obstetrical accidents or congenital abnormalities.[13] However, vitamin D is the exception as there have been interventional trials, cohort studies, and associational studies on the effects of vitamin D deficiency on fetal mineral metabolism in humans.[13] Because of the difficulties and ethics behind human data, much of the research on fetal mineral and bone homeostasis is collected from other species. There has been extensive examination using surgical, genetic, and pharmacological approaches on species such as pigs, lambs, sheep, and rodents.[13] Global and conditional genetic models have been crucial as they have enabled researchers to study PTH, calcitriol, VDR, FGF23, and other hormones. Data from these models has allowed researchers to theorize how fetal mineral metabolism and bone development are regulated in humans.[13] The following section will describe what is currently known about fetal bone development and mineral homeostasis and the importance of continuing this research for our understating of this topic.

1.2.1 Role of fetal skeleton, kidneys, and intestines

The kidneys and intestines play little role in mineral acquisition and homeostasis during fetal development. The amount of mineral excreted into the amniotic fluid by the kidneys and absorbed by the intestine is negligible since the fetuses only source of mineral acquisition comes from ingesting its own amniotic fluid. While the fetal skeleton does play a role through its contribution to the maintenance of serum calcium through bone turnover, it is likely that it is not contributing to the same extent during normal fetal development as it does after birth. This is

likely because the flow of minerals during fetal development is mainly into the fetal skeleton rather than equal inflow and output during the normal bone turnover in adults. After birth, the intestines become the major source of minerals, the kidneys begin to reabsorb mineral, and normal bone turnover of the skeleton contributes additional mineral to the circulation.[13] Since these organs have a minimal role during fetal development the fetus must rely on another source for mineral acquisition, namely the placenta.

1.2.2 The placenta

The placenta is a temporary organ that exists during pregnancy and after is ejected as biological waste.[32] It connects the developing fetus to the uterus of the mother and has many functions during gestation, including oxygenation and nourishment of the developing fetus, waste product excretion, fetal protection against the maternal immune system, and synthesis of various hormones and neurotransmitters.[32] The placenta is the largest fetal organ and the first to develop during mammalian development. Upon the blastocyte implanting in the uterus, the outermost layer forms the trophectoderm (this will give rise to the placenta) while the inner cell mass will later differentiate into the fetus. The trophectoderm is the first cell lineage and it will eventually give rise to the cytotrophoblast cells.[32,33] The cytotrophoblast cells are highly proliferative in nature and they quickly give rise to the trophoblast cells.[32] Trophoblast differentiation may occur via two pathways, villous or extravillous.[33] The villous phenotype is distinguished by cell fusion to the terminally differentiated multinucleated syncytiotrophoblast. This is the major element of the placental barrier and forms the outer epithelial layer of the chorionic villi.[32,33] The extravillous trophoblast cells migrate into the decidua and remodel uterine arteries allowing blood flow to the placenta (Figure 5).[33] The human placenta is a haemochorial villous organ, the

maternal blood comes into direct contact with the fetal trophoblast cells. This allows for an intimate relationship between the developing fetus and nutrients from maternal circulation.[33]

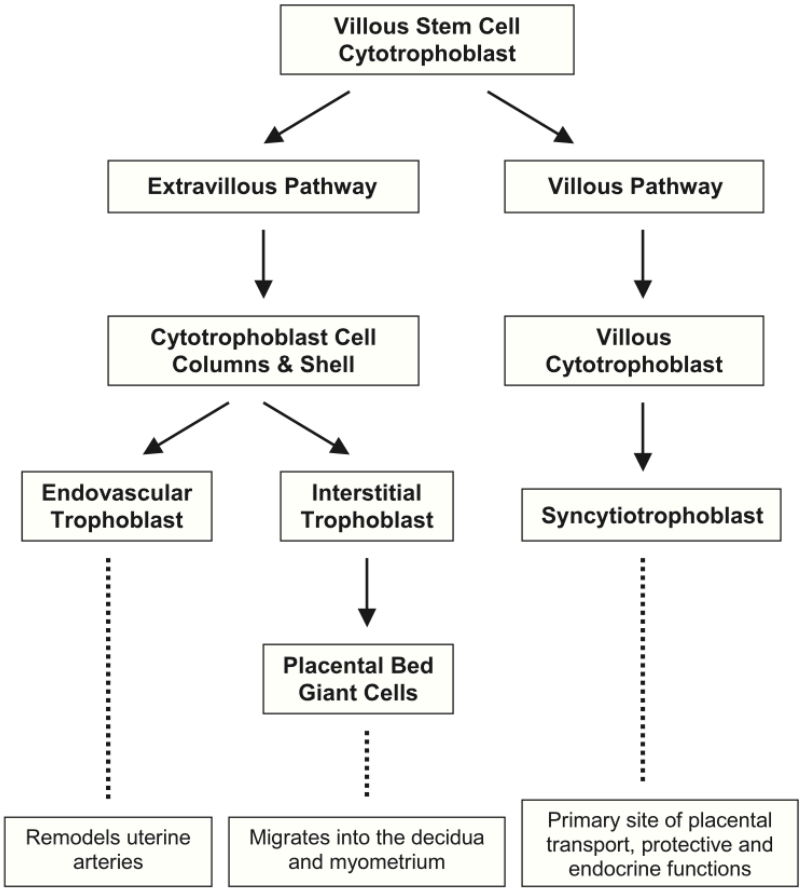

Figure 5: Pathway of trophoblast differentiation and function.[33] The cytotrophoblast gives rise to the trophoblast. These trophoblasts will differentiate via two pathways. The extravillous pathway gives rise to the endovascular trophoblasts and interstitial trophoblasts. The villous pathway gives rise to the syncytiotrophoblasts. Used with permission from Elsevier © 2004 (Appendix C).

Because of the ethical issues of studying human placentas, animal models are used for research. The laboratory mouse is one of the commonly used models to study the placenta because of its similarities with the human placenta.[34] The murine model, like that used in the research of this thesis, have placentas that have very similar structure to the human placenta (Figure 6). They are discoid in shape and have a haemochorial inner structure.[13,34] Murine placentas also have similar functional and diffusional characteristics to human placentas making murine model a great way to gain insight by studying murine genetic knockout models of *in utero* fetal loss.[34] While the similarities between mouse and human placenta makes them a great model for studying the placenta, they do differ in some respects. While they are functionally analogous, murine placentas have a labyrinth whereas human placentas have a villous structure.[35] However both areas contain the maternal and fetal blood channels pictured in Figure 6 and Figure 7 below. The murine placenta also has the intraplacental yolk sac (Figure 7), an important site of exchange between maternal and fetal circulations, that is not found in the human placenta.[36] Even though there are differences, the murine placenta is still a great model because of its similarities to the human placenta. However, as with any animal model there are limitations, and we must be conscious of the fact that what is concluded from these models may not be the case in humans.

The fetus has several developmental goals, including maintaining higher extracellular mineral concentrations compared to maternal circulation and adequately mineralizing the skeleton before birth.[13] One of the main roles of the placenta, with respect to bone and mineral metabolism, is to actively pump minerals from the maternal to fetal circulation. The placenta can keep up with the high fetal demand and provide the fetus with nutrients from maternal circulation even if the maternal concentrations are low.[13] Calcium is likely actively transported across the placenta

through mechanisms that are like those within the renal and intestinal cells. Gated calcium channels [such as transient receptor potential cation channel subfamily V member 6 (TRPV6)] open within the maternal-facing basement membranes allowing for calcium entry. This calcium is then shuttled to the opposite basement membrane bound to binding proteins, such as calbindin-D-9k, a product of the gene *S100g*. Then finally Ca^{2+}-ATPase at the fetal-facing basement membranes actively pumps the calcium into fetal circulation.[13]In the placenta the calciotropic genes are expressed in the trophoblasts and most highly expressed in the intraplacental yolk sac cells in rodents.[13] The mechanisms for phosphorus and magnesium transport across the placenta have not yet been established.[13]

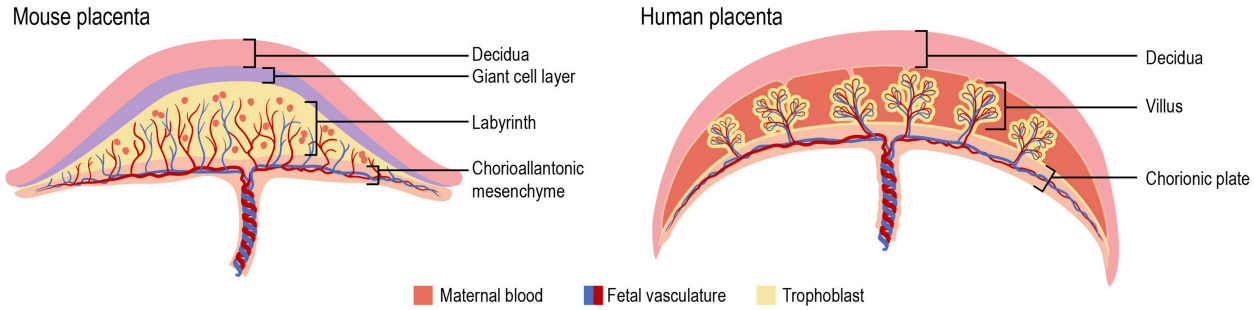

Figure 6: The structure of the murine placenta and human placenta.[36] Used with permission from Elsevier © 2020 (Appendix D).

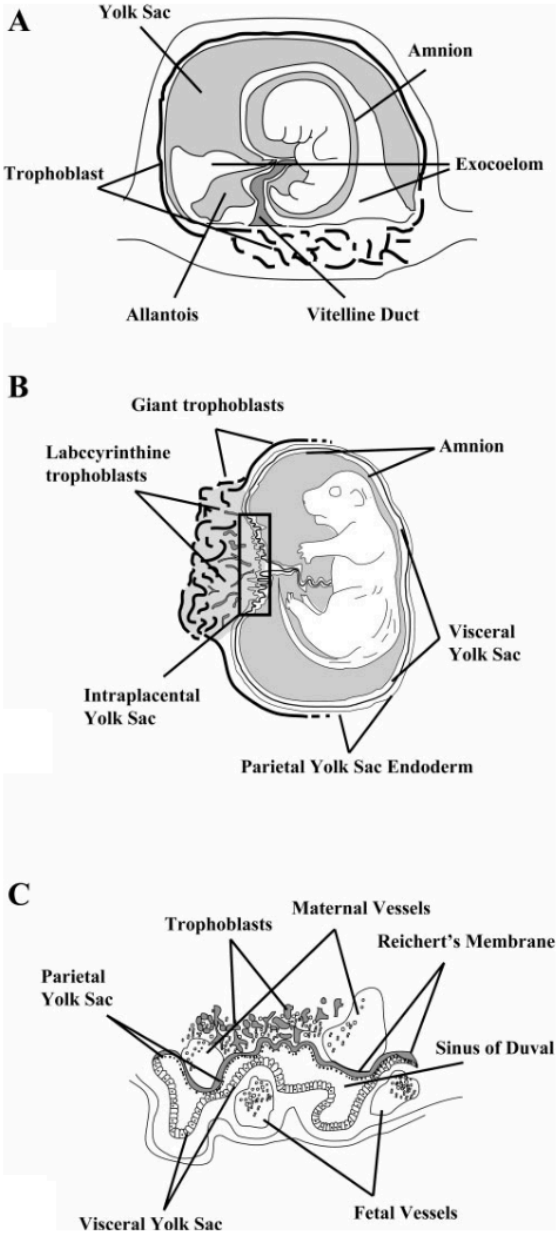

Figure 7: Formation and development of rodent placenta including the intraplacental yolk sac.[37] (A) Showing the placenta and fetal at early stage of development. (B) The fetus and placenta towards late pregnancy. (C) Magnified section of the placenta from figure (B) that displayed the yolk sac. Used with permission from The American Physiological Society © 2002 (Appendix E).

1.2.3 Fetal Bone Development

During human fetal bone development, the pattern for the embryonic skeleton is laid down at 8 weeks post fertilization.[13,38,39] However, rapid bone formation and mineralization do not occur until later stages of fetal development, mostly in the third trimester, causing the need for extremely high mineral acquisition.[13] The initial pattern starts with the migration of mesenchymal cells derived from embryonic lineages. The cells will migrate to the sites of future bone, forming the layout of the skeleton.[39,40] At these sites they form condensation of high cellular density. Within these condensations, the mesenchymal stem cells will either differentiate into chondrocytes or osteoblasts, depending on what ossification process the site of bone will undergo.[39,40] Ossification is one of the most important steps of bone development and growth. It involves the coordination of osteoblast differentiation, matrix production, mineralization, and vasculogenesis.[38] In certain areas, the mesenchymal cells differentiate directly into osteoblasts to form bone, such as in the membranous neuro- and viscerocranium and part of the clavicle. This direct bone formation is known as intramembranous bone formation or intramembranous ossification.[39] In other areas of the developing skeleton, the mesenchymal cells that differentiate into chondrocytes will form cartilaginous models of future bones, including the skull base and the posterior part of the skull, the axial skeleton, and the appendicular skeleton. This cartilage will be mineralized into bone later through endochondral bone formation/endochondral ossification.[40]

1.2.3.1 Endochondral Ossification

Most bones, such as the long bones of the limbs, develop through the process of endochondral ossification. This process starts with a cartilage model that later during fetal development and

postnatal growth, up until puberty, gradually gets replaced by mineralized bone.[40] Much of what is currently known about skeletal development comes from studying murine models.[13] Human skeletal development is similar to rodent skeletal development, but due to ethics the human skeleton has not been studied as extensively as the animal models. Therefore, the following section describes what occurs specifically in mouse bone, however human bone likely develops in a similar manor. The chondrocytes are very specifically arranged with the cartilage in morphologically distinct zones, that reflect the changes in the functional state of the cells. There are four main chondrocyte zones (Figure 8). These zones allow the cells to go through well-ordered and controlled phases of proliferation, differentiation, and apoptosis.[13,40,41] The first zone, furthest from the ossification front, is the resting zone. The next zone is the proliferation zone where the chondrocytes divide rapidly. The round proliferating chondrocyte will flatten and move into the third zone known as the adjacent resting zone, where they are called pre-hypertrophic chondrocytes. And finally, they move into the fourth zone, where these chondrocytes will undergo hypertrophy, where they will increase in volume and secrete extracellular matrix and eventually becomes mineralized. This process is what allows bone elongation to occur. The rate the chondrocytes go through the four zones influences the proper elongation of the bones and sets the timing for the methodical replacement of cartilage matrix with bone matrix.[41] Once in the fourth zone the chondrocytes will undergo apoptosis, the cartilage matrix surrounding them also breaks down, allowing space for the cells from the ossification front to move in.[40]This includes blood vessels, osteoclasts, precursors of osteoblasts and bone marrow cells. The osteoclasts that invade will assist in the breakdown of the cartilage matrix and the osteoblasts will differentiate and use the remnants of the cartilage matrix as a pattern for the deposition of the bone matrix.[40] This process starts first in the centre in the area

known as the primary ossification centre and later at each end in the areas known as the secondary ossification centres (Figure 9).

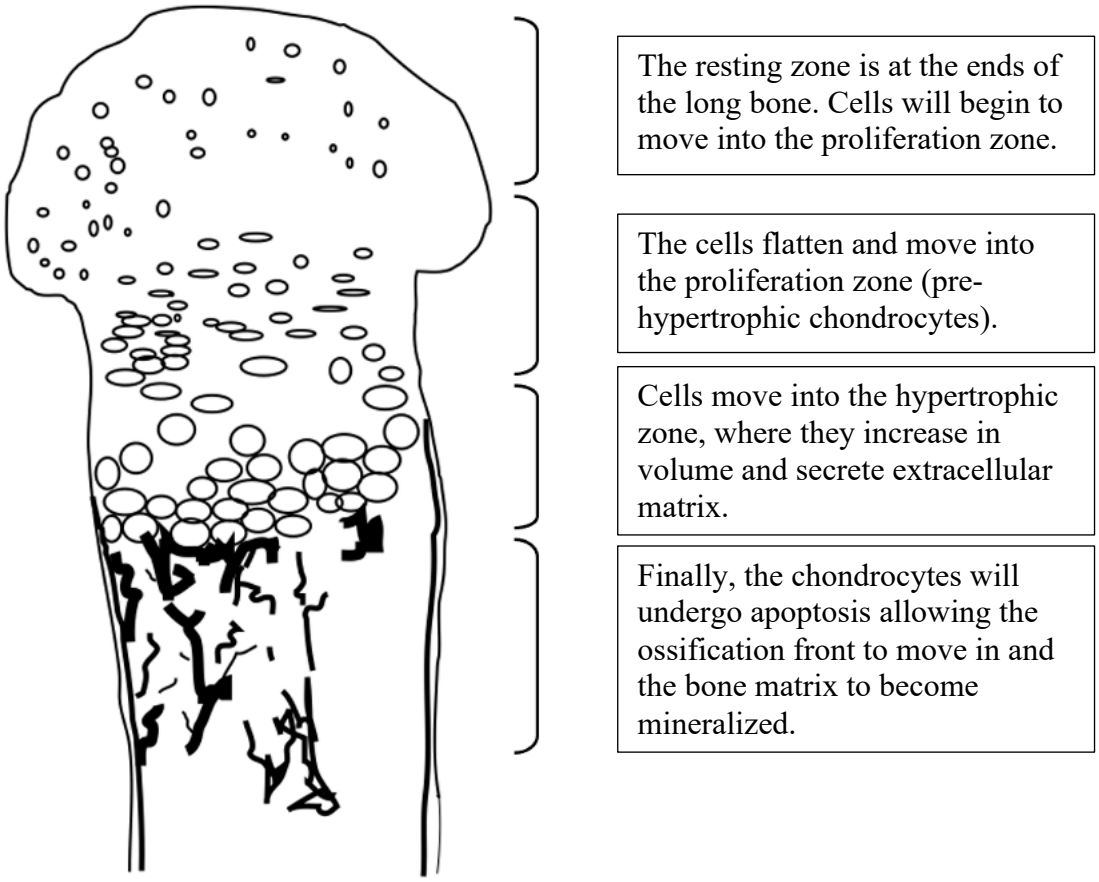

Figure 8: The four main chondrocyte zones during endochondral ossification in mouse bone. Reference photo adapted from Dr. Christopher Kovacs.[42] Used with permission from Elsevier © 2018 (Appendix F).

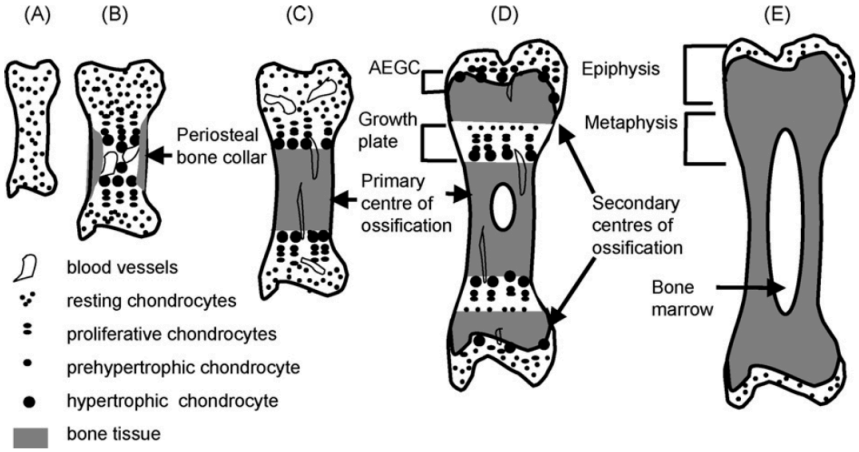

Figure 9: Endochondral ossification in long bones, such as the tibia, during fetal development.[40] As the chondrocytes move through the zones, the bone elongates. This process ends when the growth plates fuse after puberty. Used with permission from The International Journal of Biochemistry & Cell Biology © 2008 (Appendix G).

Chondrocytes in the ossification centres are influenced by several extracellular factors, including Indian Hedgehog (Ihh) and PTHrP. Ihh is a secreted factor expressed by the pre-hypertrophic chondrocytes (Figure 10).[40] It stimulates chondrocyte proliferation, and it inhibits chondrocyte hypertrophy through its induction of the expression of PTHrP.[40] PTHrP is expressed locally by early proliferating chondrocytes and acts on the PTH1R in the growth plate (Figure 10). PTHrP maintains chondrocytes in a proliferative state and prevents terminal hypertrophy.[40] This will be discussed in greater detail in section 1.2.4.4.

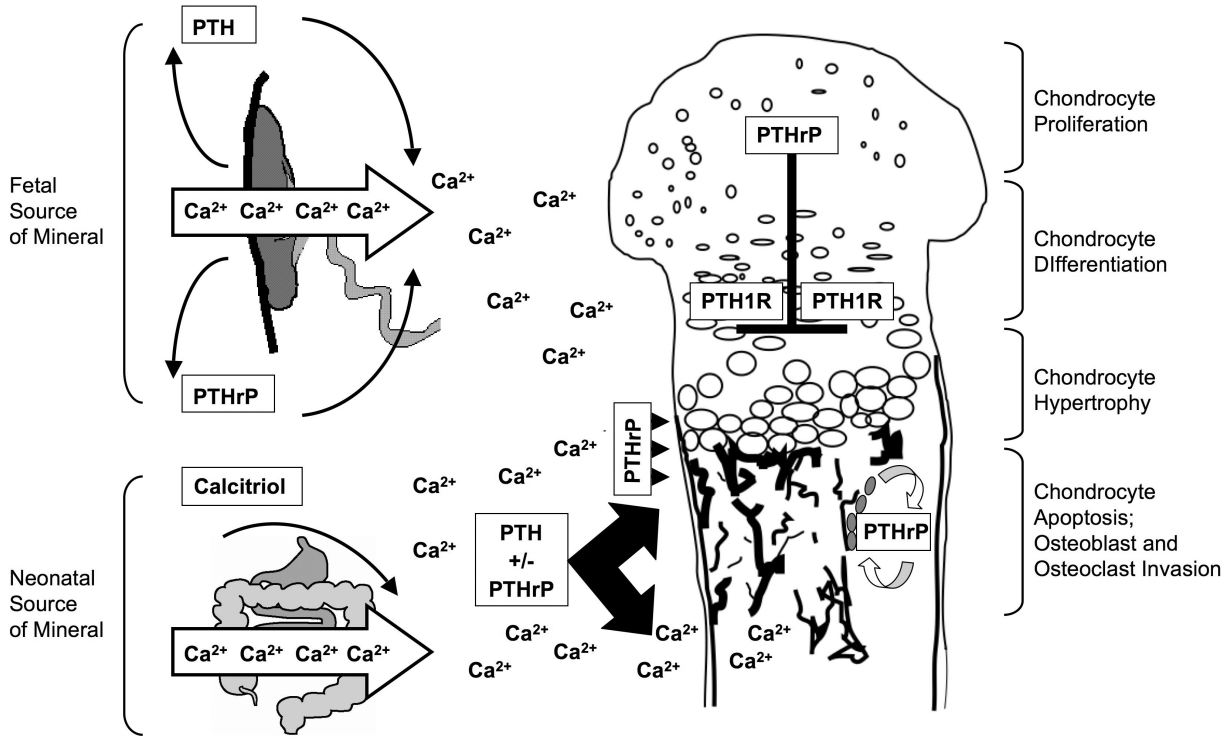

Figure 10: Expression of PTHrP and where it acts during endochondral ossification.[42]

Mineral supply is increase by the placenta, PTHrP and PTH (during gestation) and by the intestine calcitriol (during the post-natal period) for the developing/growing long bones. It is the local PTHrP from the early proliferating chondrocyte acts on the PTH1R to keep chondrocytes in the proliferation stage. Used with permission from Elsevier © 2018 (Appendix F).

1.2.4 Calciotropic and Phosphotropic Hormones

1.2.4.1 PTH

PTH is the major regulator of calcium and bone metabolism in adults. Because of this it would be expected to be a main factor in fetal life but instead PTH plays a minor role in fetal mineral homeostasis and skeletal development. It circulates at lower levels in fetal rodents, lambs, and calves compared to maternal concentration.[18,43-46] This is also the case for human infants, who have levels as low <0.5 picomolar (pM), compared to maternal and adult values.[47] The low levels of PTH in fetuses can be detected as early as 19 weeks of gestation.[48] Intact PTH cannot cross the placenta from maternal circulation to fetal circulation, therefore PTH within fetal circulation is likely from fetal origin. However, despite these low PTH levels in the animal models and in humans, fetuses are hypercalcemic compared to maternal and adult values. CaSR on the fetal parathyroids are activated due to the high levels of total and ionized calcium in fetal circulation, which in turn should suppress the synthesis and release of PTH in fetal circulation.[49]

The low concentration of PTH in fetal circulation is still important for blood calcium regulation because ablation of PTH or PTH1R causes hypocalcemia in the fetal mice.[13] This is supported by a study where CaSR was inactivated in fetal mice, which led to increased levels of PTH and an increase in serum calcium.[13,50] It is through its role in regulating serum calcium concentrations in the fetus that PTH also influences the mineralization of the skeleton.[51] Some studies on *Pth* nulls suggest that it has a role in regulating bone formation in the developing fetus as the *Pth* null mice have slightly shortened tibial metaphyseal lengths, shorter metacarpals and metatarsals, smaller vertebrae, reduced trabecular bone volumes, and fewer osteoclasts and osteoblasts.[51] However, when the same mice were studied in a different genetic background, there were no alterations in

limb lengths or skeletal morphology, and only a reduction in skeletal mineralization.[51] Overall, the findings suggest that if PTH does play a role in regulating skeletal development, it is modest and dependent on genetic background for it to be noticeable. PTH may also play a minor role in the regulation of placental calcium transport since it is expressed at low levels in the placental trophoblasts.[51]

1.2.4.2 Calcitriol

Calcitriol would be expected to play an important role in fetal development because of its key role in the neonate and adult skeleton. But instead, it plays a minimal role in fetal mineral homeostasis and skeletal development. 25OHD can readily cross the placenta from maternal to fetal circulations, however, despite this, calcitriol still circulates in the fetus at values that are less than 50% of the maternal values in rodents[52-55] and pigs.[56] This was also mirrored in human infants.[57-61]

Studies on vitamin D deficient rats[62-64], *Cyp27b1* null pigs[56], and *Vdr* null mice[53,65] all displayed normal serum calcium and phosphorus concentrations, and normal skeletal ash weight and mineral content. *Cyp27b1* null fetuses[66] displayed normal serum mineral concentrations, endochondral bone development, and placental calcium transport suggesting that loss of fetal calcitriol does not have a major impact on fetal development. Human data is consistent with these findings that calcitriol may not be necessary for normal fetal bone and mineral homeostasis. Babies born with severe vitamin D deficiency also have displayed normal serum calcium and phosphorus concentrations, normal serum PTH levels, and normal skeletal morphology and mineral content.[13]

Low levels of calcitriol in the fetuses are due to several recognizable factors. Low levels of PTH causes reduced *Cyp27b1* and increased *Cyp24a1* activity, this leads to low levels of calcitriol and is also responsible for the increased levels of 24-hydroxylated metabolites in fetuses.[13] High levels of calcium and phosphorus in fetal blood will also cause low levels of calcitriol as they will suppress *Cyp27b1*.[13]

1.2.4.3 FGF23

Like PTH and calcitriol, FGF23 plays a minimal role in fetal mineral homeostasis and skeletal development. Levels of intact FGF23 in fetal circulation is similar in value to maternal circulating FGF23 during late pregnancy[67], which is increased by two-fold from female pre-pregnancy values.[68] It appears that FGF23 does not cross the placenta as *Fgf23* null fetuses have undetectable levels of FGF23 even though it is circulating in their heterozygote mothers.[67] Studies of both absence and excess of fetal FGF23 in mice have shown no difference in levels of serum calcium and serum phosphorus, amniotic fluid phosphorus, skeletal mineral content, skeletal morphology, or placental phosphorus transport.[67] These findings indicate that FGF23 does not importantly regulate fetal phosphorus metabolism.

1.2.4.4 PTHrP

Before its discovery, researchers identified that human cord blood contained elevated level of PTH-like bioactivity and low to undetectable immunoreactive PTH compared to reference values of maternal blood.[69-71] From this it was inferred that a PTH-like hormone must circulate during fetal life. PTHrP was subsequently identified from tumors that cause humoral hypercalcemia of malignancy (HHM), a condition that mimics the effects of excess PTH and resembles primary

hyperparathyroidism, but with normal levels of PTH.[69-72] With the development of bioassays, the majority of HHM patients were observed to have increased urinary levels of cyclic adenosine monophosphate (cAMP) which is highly specific for hyperparathyroidism. This indicates that the PTH receptor is activated to increase the excretion of cAMP into urine.[72] Since these patients had low to absent levels of PTH these findings suggest a humoral non-parathyroid substance was activating the renal PTH receptor. To identify this PTH-like substance assays of adenylyl cyclase activation were used, and PTH-like peptides were subsequently purified from squamous lung, breast, and renal carcinomas.[72] The tumor-derived protein has predicted isoforms of 139,141, and 173 amino acids compared to the 84 amino acids of PTH. The protein was therefor called PTHrP because it mimics the actions of PTH through a shared PTH/PTHrP receptor, known as the PTH1R.[51,69,72-74]

PTH and PTHrP genes likely arose through the duplication of a common ancestor.[51,74] They are identical in the first 8 amino acids, and the following 21 amino acids share a high degree of secondary structure. The first 34 amino acids of both peptides allow them to share the common PTH1R, and is the basis for their overlapping biological effects.[74] However, beyond the first 34 amino acids PTHrP and PTH diverge and are no longer similar. Even though the proteins share the same receptor, PTHrP largely functions as a paracrine factor or local messenger within tissues, while PTH is a circulating hormone that acts on remote target tissues.[69] There is evidence to support an alternate mid-region receptor for PTHrP which accounts for differences in the actions between PTH and PTHrP, this will be discussed in more detail in a following section of this thesis.

PTHrP has three different splice variants in humans: $PTHrP^{1-139}$, $PTHrP^{1-141}$, and $PTHrP^{1-173}$. However, in rodents there is only the full length $PTHrP^{1-139}$. PTHrP is a prohormone that is processed into several circulating peptides: $PTHrP^{1-36}$, $PTHrP^{38-94}$, $PTHrP^{38-101}$, and $PTHrP^{107-139}$.[13] It is found in many tissues including epithelia, mesenchymal tissues, endocrine glands, and the central nervous system.[69] The gene is also expressed in many fetal tissues including the skeletal growth plates, cardiac and vascular smooth muscle, trophoblasts, amnion, chorion, umbilical cord, and intraplacental yolk sac (in rodents).[13,51] This widespread distribution suggests a diversity of roles for PTHrP which are still under investigation. Previous research on the roles of PTHrP include its influence in the regulation of keratinocyte differentiations, facilitation of tooth eruption, regulation of vascular smooth muscle, local action on the developing skeleton to direct the growth and mineralization of endochondral bone, regulation of placental calcium transport, regulation of serum mineral concentrations, and renal handling of mineral excretion.[75] Loss of PTHrP causes some of the following functions including the absence of mammary tissue development beyond the bud stage,[75,76] immature lungs with low surfactant production,[75,77] and heart valve abnormalities.[75,78] The following sections will focus on what has been previously discovered on PTHrP functions and how these findings lead to the basis from this thesis project.

1.3 Human Studies on PTHrP

As mentioned above, researchers identified PTHrP from tumors that cause HHM.[69,70,72] Patients with HHM displayed some features of primary hyperparathyroidism, including hypophosphatemia, but had normal levels of PTH.[69,70,72] Since it has been identified, PTHrP has been found throughout tissues in both human adults and fetuses. It is present at high levels in the fetal circulation and produced by many tissues. In the embryo and fetus, *Pthrp* is expressed by

skeletal growth plates, cardiac and vascular smooth muscle, trophoblasts, amnion, chorion, umbilical cord, and other tissues and cell types.[13]

While there have been no humans identified with the absence of PTHrP production, suggesting that it may be embryonically lethal in humans, there have been human fetuses that have been identified to have mutations involving the PTH/PTHrP receptor (PTH1R).[73] Blomstrand lethal chondrodysplasia (BLC) is caused by inactivating homozygous or compound heterozygous mutations of the *PTH1R* which encodes for the PTH1R. The human fetuses with this mutation die *in utero* and display fetal abnormalities such as shorten limbs and low birth weight.[69,73,79] Jansen's metaphyseal chondrodysplasia (JMC) is caused by mutations in the *PTH1R*, which lead to the receptor being activated even in the absence of a ligand (PTH or PTHrP).[69,73,80] JMC is characterized by short-limbed dwarfism and severe hypercalcemia and hypophosphatemia.

1.4 Previous research on the roles of PTHrP

After the creation of specific radioimmunoassays which allowed researchers to isolate PTHrP from tumors of HHM patients, the focus then turned to understanding what its purpose was under normal circumstances. As previously mentioned, PTHrP is expressed in many adult and fetal tissues, and this widespread distribution suggests a diversity of roles.[69] To explore these roles (during both adult and fetal life) researchers have created various animal models using approaches such as the removal of the parathyroid glands and gene knockout experiments.

1.4.1 PTHrP regulates endochondral bone formation

To create a global *Pthrp* knockout, researchers disrupted the gene in murine embryonic stem cells by homologous recombination, and then introduced the null allele to the mouse germ line.[81] The goal of this model was to explore how the loss of PTHrP would influence development and growth. They first discovered that while the genotypes of the mice born were consistent with a Mendelian pattern of inheritance indicating no prenatal lethality, once born the global deletion of *Pthrp* in mice was lethal. The homozygous mice died immediately or minutes after birth likely from a combination of effects, including hypocalcemia, chondrodysplasia that creates a rigid rib cage, and lungs that are immature and stiff due to lack of surfactant which could have caused asphyxia in the null neonates.[69,77,81]

During fetal development, mice homozygous for the mutated allele displayed a phenotype compared to their WT and HET littermates, with *Pthrp* null fetuses beginning to display abnormal features as early as ED 16.5. *Pthrp* null fetal samples collected at ED 18.5 (just before birth) displayed distinct phenotypic features including a domed skull, shortened snout and mandibles, protruding tongue, shortened forelimbs and hindlimbs, and rigid ribcage (depicted in Figure 11) which define a form of osteochondrodysplasia.[81] Further histological analysis determined that the cartilaginous growth plate of the *Pthrp* null tibiae were shortened and disorganized compared to the WT littermates.[81]

Due to the skeletal abnormalities seen in these null fetuses it suggested that there was a fundamental defect in their endochondral bone formation which prompted researchers to further characterize the bones to examine the cartilaginous growth plate and endochondral bone

development. In the tibial epiphyses (growth plate), the WT littermates expressed *Pthrp* in the chondrocytes in the resting zone and in the thin zone between resting and proliferation while there was no expression of *Pthrp* in the chondrocytes of the null fetuses.[82] The growth plates in the null fetuses were decreased in thickness and disorganized with no clear junction between the proliferative zone and the hypertrophic zone. The proliferative chondrocytes in the null fetuses were reduced in number and abnormal in shape, mitotically dysfunctional, and were markedly less active in the production of cartilage-specific type 11 collagen which could be the cause of the chondrodysplasia observed in these mutant mice. There was also an increase in the amount of osteoclasts (bone cells) in the metaphases in the null mice which indicates an early transition of cartilage (chondrocytes) into bone (osteoclasts).[82] These abnormalities, along with the previously described skeletal phenotype in the *Pthrp* null mice, confirmed that endochondral bone formation was disrupted by the loss of PTHrP in mice leading to an impact in proper bone elongation.

To further investigate and characterize the abnormal growth plates and shortened bones seen in the global deletion of PTHrP, other murine models were examined. The PTH/PTHrP receptor is expressed in the prehypertrophic, hypertrophic, and proliferating chondrocytes.[83] In the global murine knockout of PTH/PTHrP receptor (*Pth1r* ablation), the null offspring displayed a similar but more severe phenotype as the PTHrP global knockout. This included all PTH/PTHrP receptor null fetuses dying by ED 14.5 in one genetic background and surviving to end of gestation in another genetic background before dying within a few minutes after birth.[83] The null fetuses with domed skulls, shortened snouts and mandibles, protruding tongues, and disproportionately short limbs with accelerated mineralization in bones that undergo

endochondral ossification. With histological examination revealing the irregular growth plates with decreased proliferating chondrocytes and early mineralization into bone matrix.[83] The abnormal phenotype of these PTH/PTHrP receptor null fetuses was similar but more severe than that of the PTHrP null fetuses which suggest that the PTH1R mediates the actions of PTHrP of the chondrocytes.

The opposite was found in mice with targeted overexpression of constitutively active PTH/PTHrP receptors which showed absent mineralization at birth of many elements that develop by endochondral ossification and at the ossification centers, severe mineralization defects of radius and metatarsal bones, and short and deformed hindlimbs.[84] These mice also had tibiae that were shorter and thicker which consisted mainly of proliferative and hypertrophic chondrocytes.[84] Similar to the constitutively active PTH/PTHrP receptors, mice with targeted overexpression of PTHrP in chondrocytes using the type II collagen promoter were small in size and had disproportionate foreshortening of the limbs and tail and displayed extreme shortening and bowing of the long bones.[85] These mice also lacked ossification centers in the majority of their normal sites in the skeleton including the limbs, skull , and axial skeleton with the majority of these areas displaying little to no ossification. Histological examination once again revealed that the long bones showed no bone formation but consisted of proliferating and prehypertrophic chondrocytes embedded in the cartilaginous matrix.[85] Overall, both overexpression of a constitutively active PTH1R and overexpression of PTHrP in the chondrocytes caused mutant fetuses to have skeletons that were largely cartilaginous at birth.[84,85] This confirms that a key skeletal role of PTHrP during fetal development is to act as a brake on the terminal differentiation of chondrocytes, thereby delaying primary bone formation.

The findings from all the models above indicate the importance of PTHrP in fetal bone development and mineralization. In the absence of PTHrP, chondrocytes become hypertrophic and undergo apoptosis early, leading to shortening of the cartilaginous templates and the long bones that arise from them. Absence of PTHrP also causes the cartilaginous portions of the ribs to become rigid, calcified bone, thereby impairing breathing. Conversely, excess PTHrP delayed the chondrocyte differentiation and endochondral ossification which caused the bone to fail to mineralize before birth. The *Pthrp* null skeletal phenotype could be rescued when there was simultaneous overexpression of PTHrP or targeted expression of a constitutively active PTH/PTHrP receptor in chondrocytes.[84] These models were created by crossing heterozygous PTHrP-deleted mice with mice that had overexpressed PTHrP or with mice that had expressed a constitutively active PTH/PTHrP receptor within chondrocytes. These crosses yielded fetuses that survived birth and the perinatal period and had relatively normal skeletons.

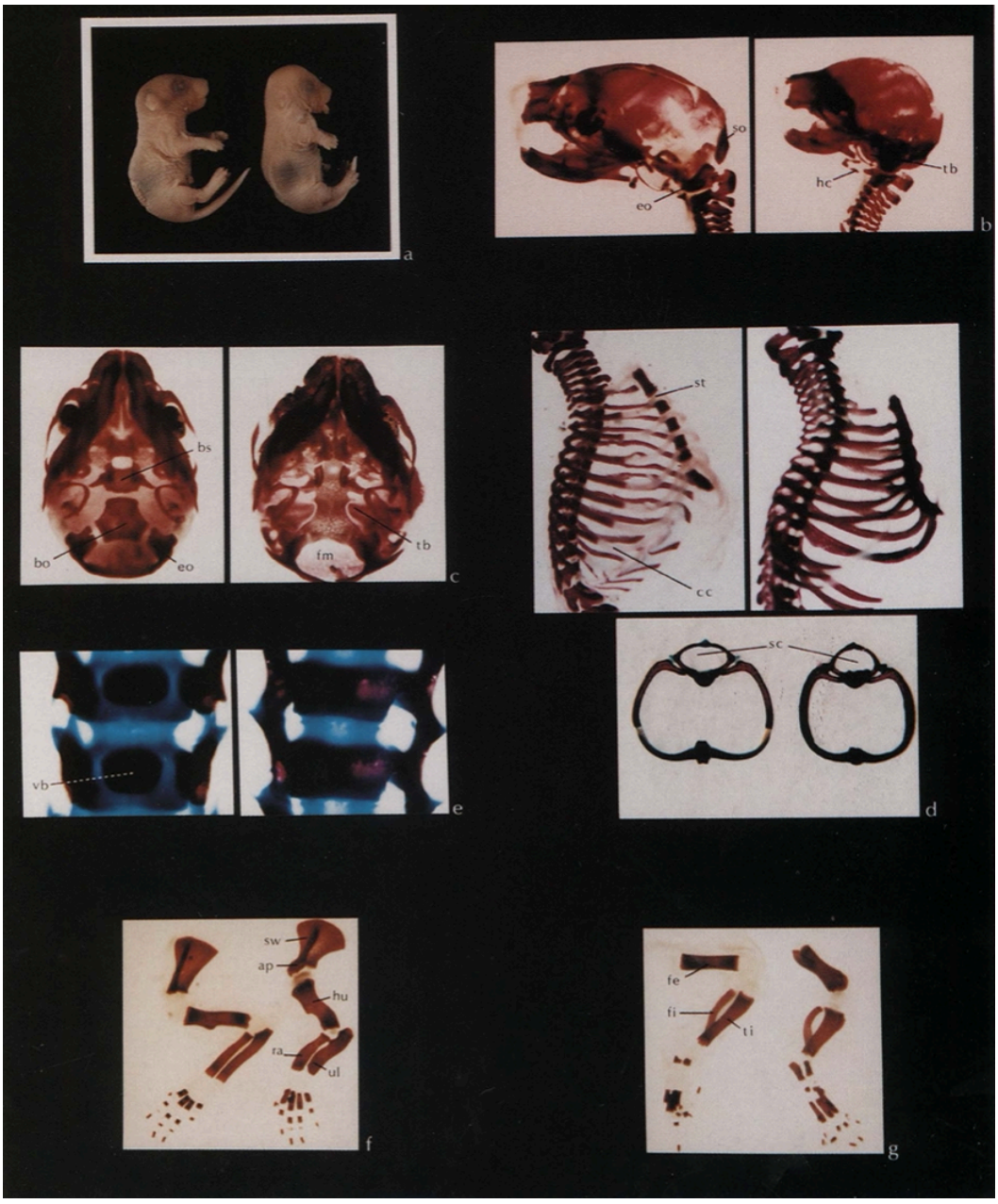

Figure 11: Skeletal abnormalities of Pthrp null mice.[81] The *Pthrp* null fetuses (samples on the right side) displayed shortened limbs and mandibles, rigid ribcage, and lethal skeletal dysplasia. Used with permission from Cold Spring Harbor Laboratory Press (Appendix H).

1.4.2 PTHrP regulates fetal mineral homeostasis and placental calcium transport

In fetal pigs, the concentration of PTH-like bioactivity and PTHrP (1-86) immunoreactivity were both higher in the fetal circulation compared to maternal values during the last three weeks of gestation.[86] The concentration of PTHrP immunoreactivity and PTH-like bioactivity decreased with increasing ionized calcium.[86] With the decrease of ionized calcium in the fetuses there was an increase in the PTHrP immunoreactivity and PTH-like bioactivity in the plasma.[86] These findings indicate that the presence of PTHrP immunoreactivity and PTH-like bioactivity in the fetal plasma was inversely related to the ionized calcium concentration in the fetus.[86] Following the discovery of lethal skeletal dysplasia and the histological impact of PTHrP, researchers focused on the physiological studies of global murine *Pthrp* knockout.[87,88] The *Pthrp* null offspring displayed hypocalcemia, hyperphosphatemia, and secondary hyperparathyroidism.[87,88] The results from the fetal pigs and these murine models, indicate that PTHrP influences mineral homeostasis in the fetus.

The null fetuses from the global murine *Pthrp* knockout, also displayed reduced placental calcium transport which was restored to normal when fetuses were treated with mid-molecular lengths of PTHrP in utero, while PTH and N-terminal PTHrP had no effect.[87] However, there was no reduction in ash weight or mineral content, but instead it was normal to slightly increased due to early and abnormal bone mineralization.[88] In the PTH/PTHrP null fetuses placental calcium transport was increased in this model due to high levels of circulating PTHrP acting on the intact mid-region receptor.[83] They had increased expression of *Pthrp* found in the placenta and to a lesser extent in the liver.[83] They also displayed an increase in circulating PTHrP and placental calcium transport. The finding from the above models supports that PTHrP influences

fetal mineral homeostasis. However, what remains to be determined is where this PTHrP is coming from.

1.4.3 The source of circulating PTHrP influencing mineral homeostasis

As described above PTHrP has a hormonal role in regulating mineral homeostasis in the fetus but the source of circulating PTHrP influencing mineral homeostasis remains to be elucidated. As PTHrP is expressed in numerous tissues during embryological and fetal development it is hard to pinpoint what tissue (or multiple tissues) is the source in fetal circulation.

One of the first potential sources studied were the parathyroid glands. In sheep that had undergone a parathyroidectomy, placental calcium transport was sharply reduced, and the fetal lambs developed hypocalcemia. However, whether the hypocalcemia observed was due to a reduction in circulating levels of PTHrP was not confirmed during this study.[13,69] The reduction in the rate placental calcium transport could be increased by infusion of mid-molecular lengths of PTHrP, but not infusions of amino- terminal fragments of PTHrP or PTH.[13] These findings suggest the fetal parathyroid glands, at least in sheep, could be a potential source of circulating PTHrP, as without them mineral transport and homeostasis is disrupted and with injections of it mid-molecular region it can reverse the rate of placental calcium trasnport.[69]

However, the fetal parathyroids are evidently not the source of circulating PTHrP or the regulator of placental calcium transport in rodent or pig models. In murine models with the genetic ablation of parathyroids (*Hoxa3* null and the *Gmc2* null fetuses) loss of the parathyroids did not alter the plasma concentration of $PTHrP^{1-34}$ nor did it reduce the rate of placental calcium

transport in fetuses.[13,18,83,88] However, in mice with the global knockout of PTHrP there was a decrease in placental calcium transport and in the PTHr/PTHrP knockout the mice had increased placental calcium transport. Mice with the PTH/PTHrP receptor knockout also showed an 11-fold increase in the concentration of $PTHrP^{1-34}$ and increased expression of PTHrP mRNA and protein in the placenta.[13] The increased expression of PTHrP in the placenta is an indicator that it is a possible source for the circulating PTHrP which influence fetal mineral homeostasis. Another study in fetal rats found that PTHrP mRNA was not detected in fetal rat parathyroids by *in-situ* hybridization or RT-PCR supporting that the parathyroids are not the source of circulating PTHrP.[89] Finally, in pigs, venous umbilical PTHrP levels are higher than the umbilical arterial levels which suggests that the placenta may be an important source of circulating PTHrP in the fetus.[13]

While the studies of fetal sheep suggest that circulating PTHrP comes from the parathyroid glands, the evidence from rodent and pig models indicate instead that the placenta is a potential source of PTHrP in fetal circulation, which in turn is influences fetal mineral homeostasis. The idea that the placenta is the dominant source of circulating PTHrP is the basis for the following research project. We set out to determine if circulating PTHrP derives from the placenta and controls systemic mineral concentrations and placental calcium transport.

1.5 Rationale, Purpose, Hypothesis, and Objectives of the Study

From the findings of the animal models previously described it should be clear that PTHrP has four distinct roles during fetal development (bone development, bone mineralization, mineral homeostasis, and placental calcium transport). These four roles can be grouped into paracrine

versus hormonal functions of PTHrP. Its paracrine function includes actions of the locally produced PTHrP to regulate chondrocytes within growth plates and, thereby, skeletal development. Its hormonal function may be regulating serum mineral concentrations and, thereby, providing the mineral needed for bone mineralization. Placental calcium transport may be either paracrine (if PTHrP is produced locally in the trophoblast cells) or hormonal (if it is regulated by circulating PTHrP).

While it is evident that PTHrP is responsible for all these different roles, the source of the circulating PTHrP has not yet been identified. The role of placental PTHrP in fetal skeletal development and mineral homeostasis is also uncertain. Previous research in rodent models suggests that placental PTHrP plays a role in fetal mineral homeostasis and that the placenta may be the source of circulating PTHrP. Therefore, the goal of this research is to determine the role of placental PTHrP in regulating fetal mineral homeostasis and skeletal development.

We hypothesized that selective ablation of *Pthrp* from trophoblasts would lead to fetal hypocalcemia, hyperphosphatemia, reduced placental calcium transport, low renal calcium excretion into amniotic fluid, and reduced skeletal mineral content. To achieve this goal, we created a conditional knockout of *Pthrp* in the trophoblasts of fetal mice. This allowed us to study the consequences of removing PTHrP from the placenta, without the confounding effects of loss of PTHrP in the skeleton.

The objectives of this research project were to determine whether, by the end of gestation, loss of placental PTHrP:

1. Reduces fetal serum minerals (calcium and phosphorus)
2. Reduces skeletal ash weight and mineral content (calcium, phosphorus, and magnesium)
3. Alters endochondral bone development
4. Reduces placental calcium transport
5. Alters expression of genes within the placenta that are involved in placental calcium transport

II. Materials and Methods

2.0 Animal husbandry

2.0.1 Murine model

The floxed *Pthrp, Pthrp*$^{flox/flox}$, mice were provided by Dr. Andrew Karaplis at the Lady Davis Institute of McGill University in Montreal, Quebec.[90] In this model two *loxP* sites flank exon 4 of the *Pthrp* gene.[90] The *Tpbpa/Ada-Cre*$^{+/+}$ mice, that enable trophoblast-specific excision of DNA within two *loxP* sites, were provided by Dr. Yang Xia of the University of Texas at Austin, (TX,USA). Trophoblast-specific protein α, Tpbpa, and adenosine deaminase, Ada, were assembled as a chimeric Tpbpa/Ada enhancer. Dr. Xia's lab demonstrated that combining a single Tpbpa enhancer with a single Ada enhancer created a more efficient trophoblast (placenta) specific expression of Cre. This chimeric enhancer arrangement was used to drive the expression of the Cre recombinase transgene in the trophoblast of transgenic mice.[91]

The following crosses were performed to maintain the colonies we received from Dr. Karaplis and Dr. Xia's laboratories. *Pthrp*$^{fl/fl}$ males and females were crossed to maintain the floxed *Pthrp* mice (Figure 12). Tpbpa/ *Ada*-Cre$^{+/+}$ males and females were crossed to maintain the trophoblast-specific Cre mice (Figure 13). Both colonies were backcrossed annually and reselected for genotype to C57BL/6 wild-type mice from Charles River Laboratory to ensure no genetic drift in the colonies. All mice were mated at 8-10 weeks.

To create the experimental animals, we first cross the *Pthrp*$^{flox/flox}$ and the *Tpbpa/Ada-Cre*$^{+/+}$ colonies together. We crossed *Pthrp*$^{flox/flox}$*; Ada* Cre$^{-/-}$ mice to the Tpbpa/ *Ada*-Cre$^{+/+}$ mice to give *Pthrp*$^{flox/+}$*; Ada* Cre$^{+/+}$ offspring, both male and female (Figure 14). We then used the female

Pthrp$^{flox/+}$*; Ada* Cre$^{+/+}$ mice and crossed them to male *Pthrp*$^{flox/flox}$*; Ada* Cre$^{-/-}$ mice. The litters from this cross were the experimental offspring used for the study. The four fetal genotypes were: *Pthrp*$^{flox/flox}$*; Ada* Cre$^{+/+}$, *Pthrp*$^{flox/flox}$*; Ada* Cre$^{-/-}$, *Pthrp*$^{flox/+}$*; Ada* Cre$^{+/+}$ and *Pthrp*$^{flox/+}$*; Ada* Cre$^{-/-}$ (Figure 15). All mice were mated at 8-10 weeks.

The following genotype abbreviations will be used in this thesis for simplicity:

Pthrp$^{flox/flox}$*; Ada* Cre$^{-/-}$ will be *Pthrp*$^{fl/fl}$;

Pthrp$^{flox/flox}$*; Ada* Cre$^{+/+}$ will be Cre-*Pthrp*$^{fl/fl}$;

Pthrp$^{flox/+}$*; Ada* Cre$^{-/-}$ will be *Pthrp*$^{fl/+}$; and

Pthrp$^{flox/+}$*; Ada* Cre$^{+/+}$ will be Cre-*Pthrp*$^{fl/+}$.

We will only report the primary analysis of data from *Pthrp*$^{fl/fl}$ fetuses compared to Cre-*Pthrp*$^{fl/fl}$ fetuses. A secondary analysis of the dams' data will only be shown where appropriate for comparative purposes.

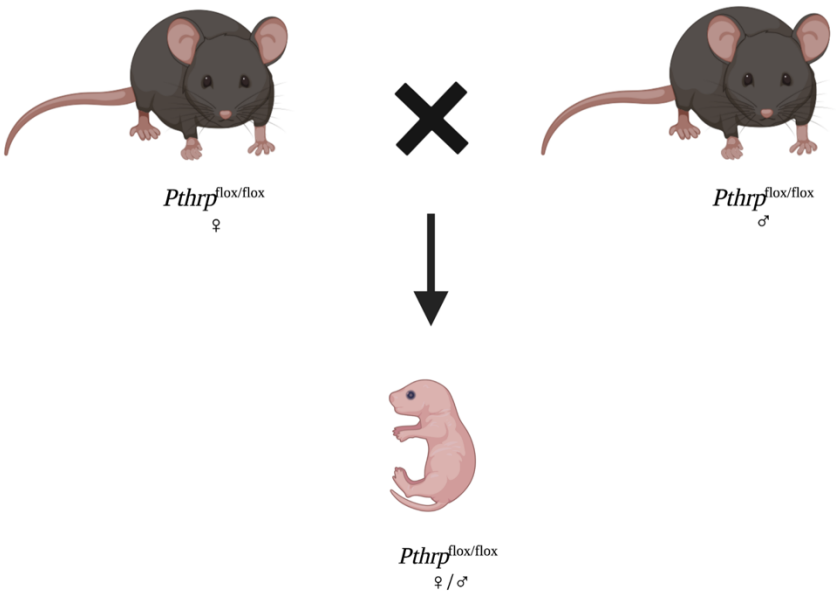

Figure 12: Mating Scheme for the maintenance of *Pthrp*$^{flox/flox}$ mice. Female *Pthrp*$^{flox/flox}$ and male *Pthrp*$^{flox/flox}$ were continuously paired. At the end of the second week females were checked for pregnancy. Pregnant females were left to deliver. All pups from this cross were *Pthrp*$^{flox/flox}$. Figure made using www.biorender.com. Permission from BioRender (Appendix I).

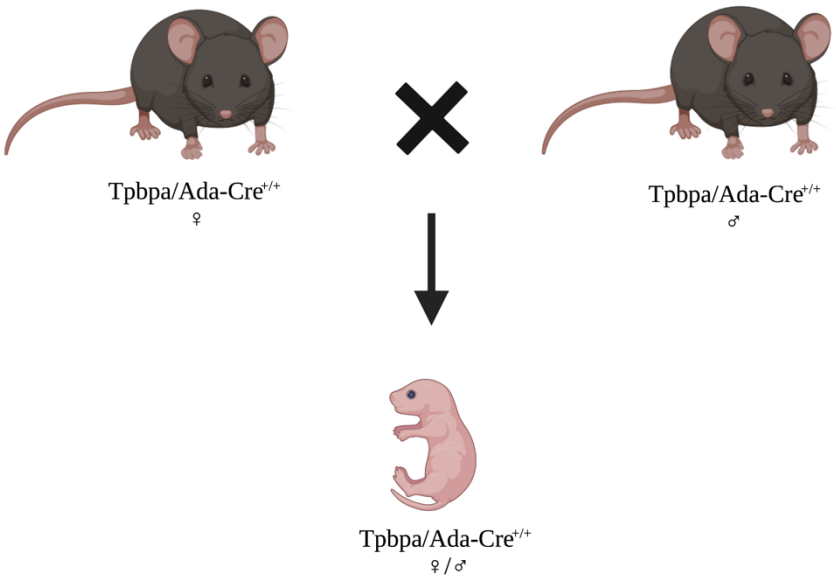

Figure 13: Mating Scheme for the maintenance of Tpbpa/Ada-Cre$^{+/+}$ mice. Female Tpbpa/Ada-Cre$^{+/+}$ and male Tpbpa/Ada-Cre$^{+/+}$ were continuously paired. At the end of the second week females were checked for pregnancy. Pregnant females were left to deliver. All pups from this cross were Tpbpa/Ada-Cre$^{+/+}$. Figure made using www.biorender.com. Permission from BioRender (Appendix J).

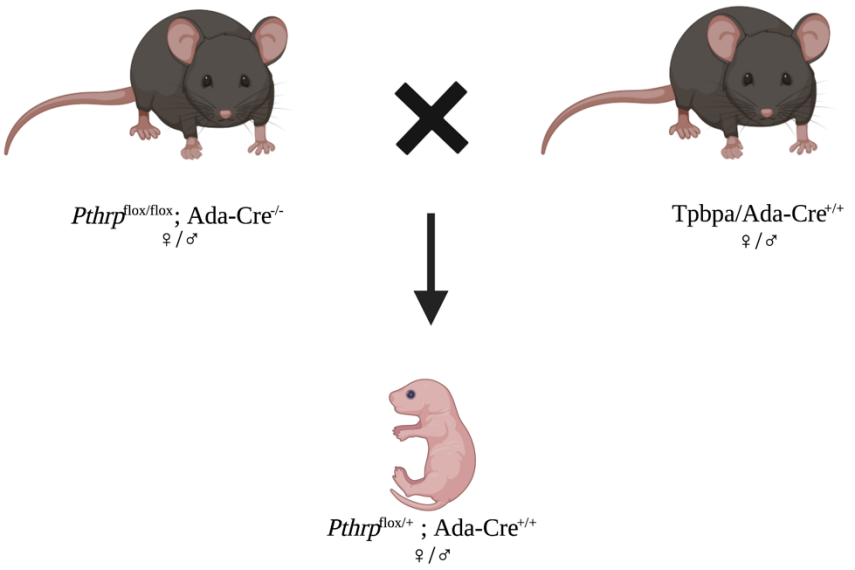

Figure 14: Mating Scheme for the cross of the *Pthrp*$^{flox/flox}$ colony with the Tpbpa/Ada-Cre$^{+/+}$ colony. Mice from the *Pthrp*$^{flox/flox}$ colony and mice from the Tpbpa/Ada-Cre$^{+/+}$ colony were continuously paired. At the end of the second-week females were checked for pregnancy. Pregnant females were left to deliver. All pups from this cross were *Pthrp*$^{flox/+}$; Tpbpa/Ada-Cre$^{+/+}$. These mice are heterozygous for the floxed allele but were all Cre-positive. Only the females were used for the experimental mating that follows. Figure made using www.biorender.com. Permission from BioRender (Appendix K).

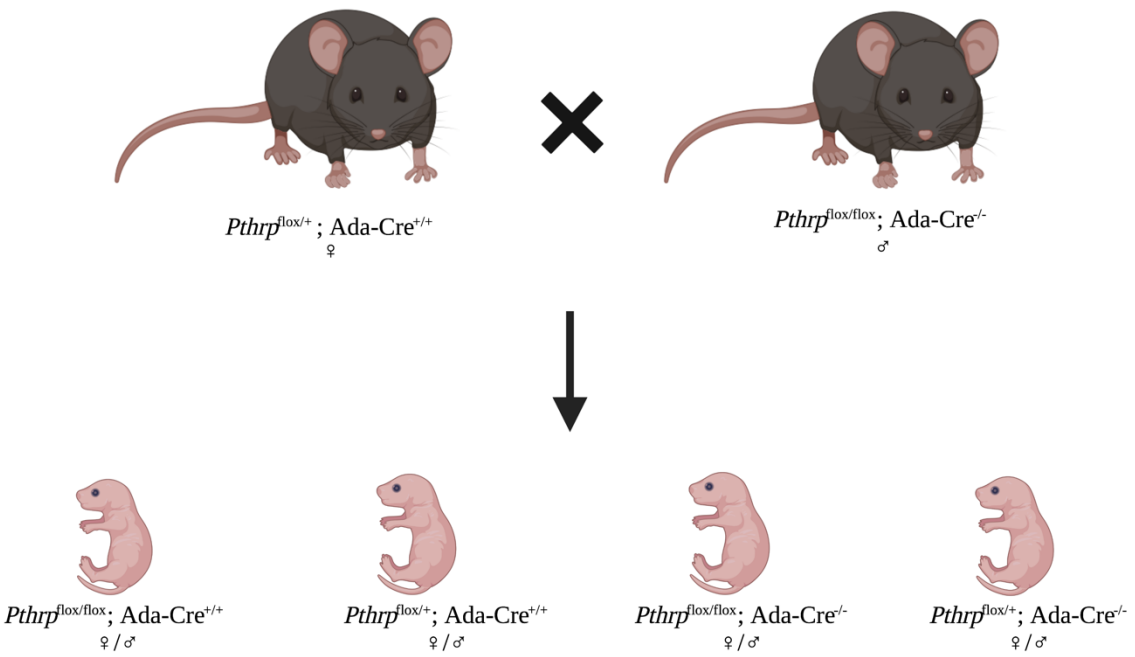

Figure 15: Mating Scheme for the experimental cross of female *Pthrp*$^{flox/+}$; Tpbpa/Ada-Cre$^{+/+}$ to male *Pthrp*$^{flox/flox}$; Tpbpa/Ada-Cre$^{-/-}$. The females from this cross were euthanized using cervical dislocation at embryonic day (ED) 18.5 and pups were harvested by c-section. The fetal genotypes were *Pthrp*$^{flox/flox}$ and *Pthrp*$^{flox/+}$ with half of each expressing Cre. The experimental group, Cre-*Pthrp*$^{flox/flox}$, was compared to the *Pthrp*$^{flox/flox}$. Figure made using www.biorender.com. Permission from BioRender (Appendix L).

2.0.2 Animal Care Approval and Animal Housing

All experimental procedures were approved by the Institutional Animal Care Committee (IACC) of Memorial University of Newfoundland, 21-02-CK (Appendix M). Animals were housed in a facility operated by Animal Care Services of Memorial University of Newfoundland. Animals were housed in individually ventilated rodent cages with 501cm^2 floor area (GM500, Techniplast Canada) and Bed-O-Cobs corn cob absorbent bedding (The Andersons, Maumee, OH, USA). This is in accordance with the Canadian Council on Animal Care (CCAC). Regular light and dark cycle were used with light from 7:00 hours to 19:00 hours. The humidity in the animal room was kept between 40% to 60% and the room temperature was kept between 22 degrees Celsius to 25 degrees Celsius. The mice were provided with food, 2018 18% Protein Teklad Global Rodent Diet® (Envigo, Appendix N), and water *ad libitum.*

2.0.3 Timed Mating

In the evening, pairs of female Cre-*Pthrp*$^{fl/+}$ mice were placed into a cage with a lone male *Pthrp*$^{fl/fl}$ mouse. They were left together overnight to mate and were separated the following morning. The female mice were checked for a vaginal mucus plug following mating. If a plug was found it signified ED 0.5 and the female was monitored for pregnancy over the following two weeks. Pregnant female mice were harvested on ED 18.5.

2.1 Genotyping

2.1.1 Animal identification

At weaning (21 days of age) mice were separated from their mother and grouped by sex. Males and females were housed separately, with a maximum of 4 mice per cage. Male mice were

housed individually for mating purposes, once they reached sexual maturity (6 weeks of age). For identification purposes, mice were briefly anesthetized with Isoflurane (Baxter, Deerfield, IL), and their right ear was crimped with an ear tag.

2.1.2 Tail sample collection

While still under anesthetic from the tagging procedure, a 0.2cm section from the end of the tail was clipped from each mouse using a sterile razor blade. Tail snips were placed into labelled 1.5 mL Eppendorf tubes. To digest tail clippings for genotyping, 300µl of Cell Lysis Solution (Qiagen, Toronto, ON) and 1.5µl of Proteinase K (ThermoFisher Scientific, Burlington, ON) was added to each tube and vortexed gently. Tubes were placed in an incubator (ThermoFisher Scientific, Burlington, ON) for 18 to 24 hours at 55°C. Following the incubation period, tails were fully digested and ready for DNA extraction.

2.1.3 DNA extraction

DNA extraction was completed following the procedure provided by Qiagen Purgene® A Kit (Qiagen, Toronto, ON). The procedure from the kit was as follows. 100µl of Protein Precipitation Solution (Qiagen, Toronto, ON) was added to the tubes containing the digested tails. Samples were vortexed for 20 seconds followed by 3 minutes in a centrifuge (ThermoFisher Scientific, Burlington, ON) at 16,000 x *g*. The supernatant was transferred into a new 1.5mL Eppendorf tube containing 300µl isopropanol. Tubes were labelled accordingly and mixed by gently inverting 50 times before being centrifuged for 1 minute at 16,000 x *g*. The supernatant was drained from the tube and discarded, leaving the DNA pellet behind. 300µl of 70% ethanol was added to the tube. Tubes were inverted several times to wash the DNA pellet

before being centrifuged for one minute at 16,000 x *g*. The supernatant was drained carefully from the tube and discarded, leaving the DNA pellet. 100µl of Hydration Solution (Qiagen, Toronto, ON) was added to samples from adult mice tails and 200µl Hydration Solution (Qiagen, Toronto, ON) was added to samples from fetal mice tails. Samples were incubated in a heat block (ThermoFischer Scientific, Burlington, ON) at 65°C for one hour to dissolve the DNA pellet. Once this incubation period was complete, samples were ready for Polymerase Chain Reaction (PCR) analysis. Samples were stored at 4°C.

2.1.4 PCR

To determine the genotypes of the mice, PCR was performed on the samples. Separate reactions were performed, one for *Pthrp,* the other for *Ada-Cre.* A third reaction was performed for beta-actin as a control.

A 2-primer system was used to distinguish between $Pthrp^{fl/fl}$ and $Pthrp^{fl/+}$. The primers sequences (provided by Dr.Karaplis' laboratory) used were:

PTHrP-SacI-F: 5'GAG GCT AAG CCA GGA GGA TT

PTHrP-SacI-R: 5'CCC CAT CCT CTC TCC TCT CT

The *PTHrP* PCR master mix was made with 1x PCR reaction buffer, deoxyribose nucleotide triphosphates (dNTPs- 0.2mM dATP, 0.2mM dTTP, 0.2mM dCTP, 0.2mM dGTP), primers (0.4µM *PTHrP-SacI-F,* 0.4µM *PTHrP-SacI-R*), 1.5mM $MgCl_2$, 2U/rxn Platinum Taq DNA polymerase (ThermoFisher Scientific, Burlington, ON), and distilled water (ThermoFisher Scientific, Burlington, ON) 18.0µl of the PCR master mix was aliquoted into 0.2ml PCR tubes

(ThermoFisher Scientific, Burlington, ON). 2.0µl of each DNA sample was added individually into the solution in each separate tube. PCR tubes were labelled to the corresponding DNA sample and were then placed into Bio-Rad C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, California).

The *PTHrP* PCR program consisted of 7 steps. Step 1, 95°C for 5 minutes for initial denaturation of the DNA. Step 2, 94°C for 5 seconds to denature the DNA. Step 3, 60°C for 30 seconds for annealing. Step 4, 72°C for 30 seconds for elongation of the complementary strand. Step 5, return to step 2 and repeat for 30 cycles for amplification. Step 6, 72°C for 5 minutes for the final elongation of any remaining strands. Step 7, 4 °C infinitely for storage of the PCR reaction.

A 2-primer system was used to distinguish between *Ada-Cre*$^{-/-}$ and *Ada-Cre*$^{+/+}$. The primers sequences (provided by Dr. Xia's laboratory) used were:

ADA-Cre F: 5'CGG TCT CTG AGA GCC ATC

ADA-Cre R: 5'CCC TGA ACA TGT CCA TCA

The *Ada-Cre* PCR was performed with the following program: Step 1, 95°C for 5 minutes. Step 2, 94°C for 5 seconds. Step 3, 56°C for 30 seconds. Step 4, 72°C for 30 seconds for. Step 5, return to step 2 and repeat for 30 cycles. Step 6, 72°C for 5 minutes. Step 7, 4 °C infinitely.

A 2-primer system with beta-actin was used as a control for the *Ada-Cre* mice. The primers used were:

β-ACTIN-F: 5'TGT GAT GGT GGG AAT GGG TCA G

β-ACTIN-R: 5'TTT GAT GTC ACG CAC GAT TTC C

The beta-actin PCR was performed with the following program: Step 1, 95°C for 5 minutes. Step 2, 94°C for 5 seconds. Step 3, 59°C for 30 seconds. Step 4, 72°C for 30 seconds. Step 5, return to step 2 and repeat for 30 cycles. Step 6, 72°C for 5 minutes. Step 7, 4 °C infinitely for storage.

2.1.5 Gel electrophoresis

Gel electrophoresis was used to separate and visualize the PCR products. A 1.2% agarose gel was made using 10 mL 10XTAE buffer (0.12M EDTA, 0.40M Tris, 11.5% Glacial Acetic Acid, pH 8), 90mL deionized water, 1.2g agarose powder (ThermoFisher Scientific, Burlington, ON), and 10µl of SYBR Safe DNA Gel Stain (ThermoFisher Scientific, Burlington, ON). The solution was heated to dissolve the agarose, in two one minute intervals. The SYBR-safe gel stain was added to the dissolved solution and gently swirled to combine. The solution was then poured into the gel casting tray, containing two gel combs, and allowed to polymerize for approximately 30 to 45 minutes. During this time, 4µl of gel loading dye [1mL 1M Tris, 0.03g bromophenol blue, 0.03g xylene cyanol FF, 60mL glycerol, 12mL of 0.5M EDTA, 27mL deionized water (dH20)] was added to each PCR tube.

Once the gel had polymerized, the tray was carefully removed and put in the proper orientation. Gel running buffer [90mL 10x TAE and 810mL dH20] was added to the gel electrophoresis chamber and the gel comb inserts were carefully removed. For each sample, 10µl of PCR product and gel dye mixture was pipetted into each well of the gel. The samples were run at 200 Volts for 20 minutes.

Following electrophoresis, the gel was placed under UV light using the Kodak Gel Doc System (Bio-Rad, Hercules, CA). The bands were visualized using Bio-Rad Image lab software (Bio-Rad, Hercules, CA). For *Pthrp*, a single 150bp band indicated the wildtype (WT) allele, a single band at 270bp indicated two floxed alleles, and bands at both 150bp and 270bp indicated one floxed allele and one WT allele (Figure 16). For *Ada-Cre*, a single band at 340bp indicated mice that were $Cre^{+/+}$ and no band indicated mice that were $Cre^{-/-}$. Beta-actin was used as a control with a band at 514bp (Figure 17). For beta-actin, a single band at 514bp indicated that the sample contained DNA and no band indicated that the sample did not contain DNA. Samples were all compared to the GeneRuler™ Express DNA Ladder ready-to-use (ThermoFischer Scientific, Burlington, ON) which had a size range from 100 to 5000 base pairs.

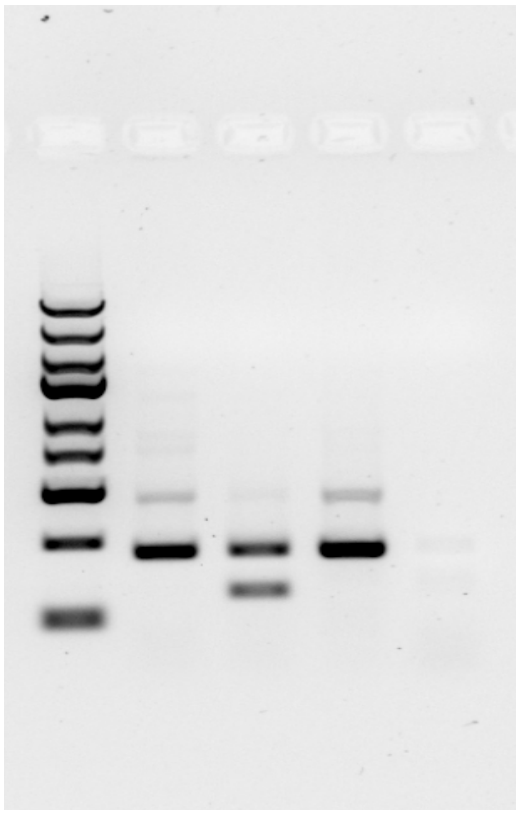

Figure 16: Gel electrophoresis of the PTHrP Floxed mice. The first well contains the GeneRuler™ Express DNA Ladder showing sizes from 100 to 5000 base pairs. The second well contains a floxed/floxed allele (single band at 270bp). This mouse was Ada-Cre negative making this the Pthrpfl/fl control mouse. The third well contains the floxed/+ allele (band at 150bp and band at 270bp) representing the Pthrpfl/+ and Cre-Pthrpfl/+ groups. The fourth well contains floxed/floxed allele (single band at 270bp). This mouse was Ada-Cre positive making this mouse the Cre-Pthrpfl/fl. Finally, the fifth well is the negative control (no band).

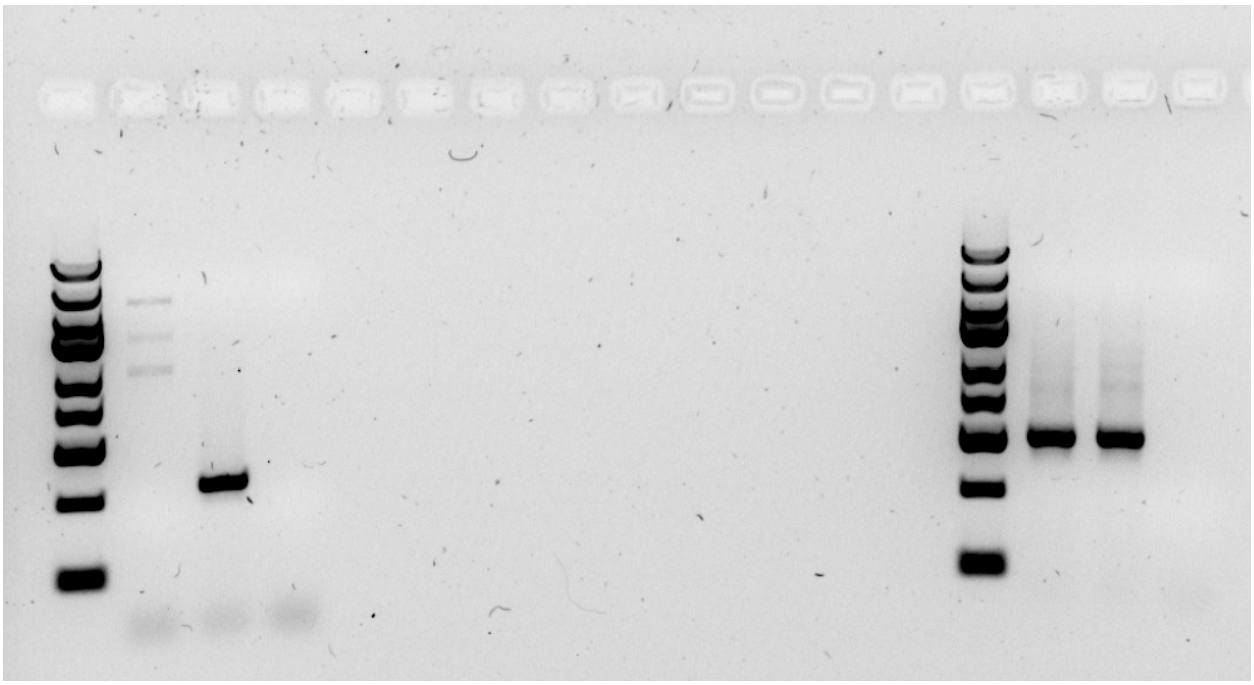

Figure 17: Gel electrophoresis of Ada-Cre mice with the Beta-Actin control. The left side of the gel contains the Ada-Cre reaction. The first well contains the GeneRuler™ Express DNA Ladder showing sizes from 100 to 5000 base pairs. The second contains the Cre-negative mouse (no band), the third well is a Cre-positive mouse (band at 340bp), and the fourth well is the negative control (no band). The right side of the gel contains the Beta-Actin reaction used to show that the samples on the right contained viable DNA. The first well is again the ladder, followed by the same two DNA samples from the Ada-Cre PCR in the second and third wells (band at 514bp), and the final well is once again the negative control (no band).

2.2 Sample collection and storage

For the sample collection for this project, all tissues were collected at the same time point, ED 18.5, the day before expected birth. The description of each collection procedure is described individually, however, during the collection process, many of the tissues were collected simultaneously from the same litter. The maternal and fetal serum and placentas were collected during every harvest along with either whole fetal bodies, or fetal tibiae.

2.2.1 Tissue Collection

On ED 18.5, fetuses were removed via Caesarean section (C-section) and detached from their placentas by severing their umbilical cords. Placentas were placed individually into labelled 1.5 mL Eppendorf tubes and then snap-frozen in liquid nitrogen to flash freeze the placentas. Tubes were transferred from the liquid nitrogen directly into a -80°C freezer to be stored for future analysis. Fetuses were euthanized via cervical decapitation. Fetal bodies were stored at -20°C for future analysis or placed in formalin for histology purposes. The tibiae were removed from the fetal bodies and placed in formalin to be sent to the Department of Histology at Memorial University of Newfoundland for paraffin embedding and sectioning.

2.2.2 Serum collection

On ED 18.5, maternal blood was collected from tail vein. A small clipping of tail was cut with a sterile razor blade and the blood was collected into a 0.6mL microcentrifuge tube (ThermoFisher Scientific, Burlington, ON). Using Isoflurane (Baxter, Deerfield, IL) mothers were briefly anesthetized before being euthanized via cervical dislocation. Fetuses were removed via C-section and detached from their placentas by severing their umbilical cords. Fetuses were kept

under a heat lamp to keep fetal bodies warm and aid in blood flow. Fetal blood was collected by making a small incision in the carotid artery and jugular vein using a sterile razor blade. Blood was collected into a 60µl Micro-Hematocrit capillary tube (ThermoFisher Scientific, Burlington, ON). Following blood collection, fetuses were immediately euthanized via decapitation. Maternal and fetal blood samples were subsequently spun using a microcentrifuge for 5-10 minutes at 16,000 x *g* to separate serum from clotted blood. Serum was carefully pipetted into individual, labelled, 0.6mL microcentrifuge tubes (ThermoFisher Scientific, Burlington, ON), and stored at -20°C for future analysis.

2.3 Analysis of serum mineral content

2.3.1 Serum and total calcium measurement

Serum and total calcium were measured using a calcium assay (Sekisui Diagnostics, Charlottetown, PEI). This assay is based on the principle that Arsenazo III reacts with calcium to form a complex that is blue purple in colour with a maximum absorbance of 650nm. Results read at this wavelength will therefore be directly proportional to the total calcium concentration in the sample. The reportable range for this kit was 0.01mmol/L to 3.75mmol/L. Serum samples were measured undiluted and following the kit protocol. A spectrophotometer (Ultraspec 2000; Pharmacia Biotech, Piscataway, NJ) was used to measure samples set at an absorbance 650nm. Deionized water was used as a blank. Samples were measured together in a single batch at the same time to control for variability from assay to assay. The absorbance of a 2.5 mmol/L calibrator was measured and then the following formula was used to calculate the calcium concentration for each sample:

Calcium (mmol/L) = $A/A_c \times$ concentration of the calibrator; where A= absorbance of the unknown and A_c= absorbance of the calibrator.

2.3.2 Serum inorganic phosphorus measurement

Serum phosphorus was measured using a Phosphorus-SL assay (Sekisui Diagnostics, Charlottetown, PEI). This assay is based on the principle that inorganic phosphorus reacts with ammonium molybdate in the presence of sulfuric acid to produce an unreduced phosphomolybdate complex. The concentration of inorganic phosphorus in the sample is directly proportional to the absorbance at 340nm. The reportable range of the kit was 0.03mmol/L to 6.46mmol/L. Serum samples were measured undiluted and following the kit protocol. A spectrophotometer (Ultrespec 2000; Pharmacia Biotech, Piscataway, NJ) was used to measure samples set at an absorbance of 340nm. Deionized water was used as a blank. Samples were measured together in a single batch at the same time to control for variability from assay to assay. The absorbance of a 2.0mmol/L calibrator was measured and then the following formula was used to calculate the inorganic phosphorus concentration for each sample:
Phosphorus (mmol/L) = $A/A_c \times$ concentration of the calibrator; where A= absorbance of the unknown and A_c= absorbance of the calibrator.

2.4 Histomorphometry

Paraffin-embedded fetal hind limbs were sectioned in 5μm thin sections and were placed onto coated slides by the Department of Histology, Memorial University of Newfoundland. Sections were then stained with 3% silver nitrate (Sigma, Kawasakai, Japan) and exposed to strong light for 30 minutes. The slides were washed, dipped (3x) in distilled water, then incubated with 2.5%

sodium thiosulfate (Sigma, Kawasaki, Japan) for 5 minutes and again washed, dipped (3x) in distilled water. The slides were then counterstained with 2% methyl green [4g methyl green (Sigma, Kawasakai, Japan) in 200µl 0.1M sodium acetate, pH 4.2] for 15 minutes. Excess dye was carefully blotted from the slides. Slides were washed in 1-butanol (Fisher Scientific, Burlington, ON) for 10 seconds (2x), and then washed in xylene for 10 seconds (2x). Using Permount (ThermoFisher Scientific, Burlington, ON) coverslips were mounted on each slide. The stained tibia sections were viewed at 5X and 10X magnification using a Leica DM500 LED Optical Microscope (Leica, Wetzler, Germany). Images of the sections were taken using a Sony DXC-S500 colour digital camera (Sony, Tokyo, Japan). Images were taken at 5X magnification. Image J 1.53A (National Institutes of Health, Bethesda, MD) was used to analyze the staining. All images were analyzed under the same manual settings for upper and lower threshold and identical area. Samples of each genotype were compared side by side to determine either the similarities or difference in the length of the growth plates and the zones within in it, the shape of the chondrocytes as they progress through the zones, and the mineralization patterns.

2.5 Fetal ash and mineral content

Individual fetuses were weighed, placed in crucibles, and reduced to ash in a furnace, Type F62700 (Thermolyne, ThermoFisher Scientific, Burlington, ON), set at 500°C for 24h. Following cremation, the ash was carefully removed from the crucibles onto a sheet of wax weighing paper using a fine paintbrush and weighed. The ash weight reflects the amount of total mineral present in the fetal skeleton. Ashed samples were then transferred into labelled acid-washed 20mL glass scintillation vials and stored at room temperature until analysis.

To prepare the samples for analysis, 253µl of 15.8N nitric acid was added to each scintillation vial containing fetal ash. Samples were left at room temperature for 5 days to dissolve and then 9.75mL of deionized water was added to each vial. Skeletal calcium and magnesium content were measured using the 2380 Atomic Absorption Spectrophotometer (Perkin- Elmer, Waltham, MA). Calcium (ThermoFisher Scientific, Burlington, ON),) and magnesium (ThermoFisher Scientific, Burlington, ON), reference standards (1mg/ml) were used to make standards within a designated range. Samples were diluted to fall within the range of these standards. Range for standards is determined at time of analysis (for this analysis range of calcium standards was 0.204M to 0.194M and range of magnesium standards was 0.5M to 1.5M). Skeletal phosphorus content was measured using a Phosphorus-SL assay (Sekisui Diagnostics, Charlottetown, PEI) as per kit protocol.

2.6 Gene expression

2.6.1 RNA extraction

RNA was extracted from the placentas (≤250mg of tissue) using the RNA® Lipid Midi Tissue Kit (QIAGEN, Toronto, ON). Tissues were homogenized and disrupted using ceramic beads (5 per tube) and QIAzol Lysis Reagent (QIAGEN, Toronto, ON) in the Percellys® Tissue Homogenizer (Bertin Technologies, Paris, FR). Homogenized samples were transferred into 15mL tubes containing 4mL of QIAzol Lysis Reagant (QIAGEN, Toronto, ON), vortexed briefly to mix, and then centrifuged at 4°C for 15 minutes. The supernatant of each sample was transferred into a clean 15mL tube and 1mL chloroform was added to each sample. The sample was shaken vigorously and then centrifuged at 4°C to separate the homogenate into aqueous and organic phases. The aqueous phase, the upper layer, was collected and placed into a new tube

with 70% ethanol was added to provide appropriate binding conditions. The sample was transferred to an RNeasy Midi column, the total RNA binds to the membrane in this column. While the phenol and other contaminates are removed by washing with buffer RW1 and with buffer RPE (2X). The RNA was eluted from the column with RNase-free water.

2.6.2 Synthesis of complementary DNA (cDNA)

cDNA was synthesized using 2μg of mouse placenta RNA using a High-Capacity complementary (cDNA) Reverse Transcription Kit (Applied Biosystems Burlington, ON). The experimental procedure followed the kit protocol. The PCR program for the synthesis of the cDNA included 4 steps carried out using Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, California). Step 1, samples at 25°C for 10 minutes. Step 2, samples at 37°C for 120 minutes. Step 3, samples at 85°C for 5 minutes. Step 4, samples were held at 4°C for infinity.

2.6.3 Real-time quantitative reverse transcriptase-PCR (RT qPCR)

RT qPCR was completed using Taqman® Gene Expression Assays (ThermoFisher Scientific, Burlington, ON) and TaqMan™ Fast Advanced Master Mix (ThermoFisher Scientific, Burlington, ON). The following were used: *S100G* (Mm00486654_m1), *Pmcal* (Mm01245805_m1), *Cyp27b1(Mm01165922_g1), Cyp24a1* (Mm00487244_m1), *Trpv6* (Mm00499069_m1), *Pth* (Mm00451600_g1), *Vdr* (Mm00437297_m1), *Ncx1* (Mm01232254_m1), and *Pdia3* (Mm00433130_m1). These assays are pre-designed and pre-optimized with a fluorogenic probe with FAM™ dye label on the 5' end and a non-fluorescent quencher on the 3'end. When targeted cDNA was amplified, the MGB probe was digested by

DNA polymerase and the FAM™ reporter dye was released. Fluorescence from free FAM™ reporter dye was detected by the real-time PCR system. The intensity of fluorescence and relative rate of amplification was related to the concentration of targeted cDNA in the sample. The thermal cycler protocol consisted of the following steps: Step 1, 50°C for 2 minutes for Uracil-N-Glycosylase (UNG) incubation. Step 2, 95°C for 20 seconds for polymerase activation. Step 3, 95°C for 1 second to denature. Step 4, 60°C for 20 seconds annealing. Step 5, Return to step 3 and repeat 40 times. 20µl Multiplex qPCR reactions (gene of interest multiplexed with reference gene) were run in triplicate wells on the ViiA™ 7 Real-Time PCR System (Fisher Scientific, Carlsbad, CA). Relative expression was determined from the threshold cycle (C_T) normalized to the reference gene (*Gapdh*). *Gapdh* was labelled with dye and was measured using a Taqman® Gene Expression Assay.

2.7 Statistical analysis

Data described above were analyzed using StatPlus: Mac Professional 2009, Build 6.0.3 (AnalystSoft Inc., Vancouver, BC). qPCR data were analyzed using the Comparative 2^{Δ} C_T method[92] and the data is presented with mean ± standard deviation (SD). For the primary analysis of biochemical and ash data compared results from Cre-*Pthrp*$^{fl/fl}$ versus the *Pthrp*$^{fl/fl}$ fetuses, and so a student's t-test was used. For secondary analysis of fetal serum calcium and phosphorus compared to their dams, one-way ANOVA (analysis of variance) was used followed by the Tukey-Kramer post-hoc test to determine which pairs of means differed significantly. All hormonal and biochemical data are presented as mean ± SD. On the graphs, significant differences are marked, and the sample size is indicated in parentheses on the x-axis.

III. RESULTS

3.0 Litter Size and Genotype Ratio

The number of fetuses was determined on ED 18.5. Litter size is an indicator of fertility and of the capacity of the mother to carry the litter to term. Litter size varied from 3 to 12 pups, with the average amount of pups per litter being 6.3. These litter sizes are typical of C57BL/6 mice[93], and we did not see any unusual signs that fetuses were being reabsorbed. The litters were in a genotype ratio of 1:1:1:1, the expected genetic ratio. Both normal litter size and the presence of all genotypes at ED 18.5 are an indication that there was no prenatal loss during gestation.

3.1 Body and Placental Weight

Body and placental weight were determined on ED 18.5. As we created a putative tissue-specific knockout of PTHrP in the trophoblast cell, we aimed to investigate how this would impact the overall development of the fetal bodies or placentas and determine whether the fetuses or their placentas would differ in size between the genotypes. We determined that there was no statistically significant difference in body weight between the Cre-*Pthrp*$^{fl/fl}$ (1.13±0.05 mg) fetuses or their *Pthrp*$^{fl/fl}$ (1.04±0.04 mg) littermates (Figure 18). There was no statistically significant difference in placental weight between the Cre-*Pthrp*$^{fl/fl}$ (0.097±0.007 mg) or Cre-*Pthrp*$^{fl/fl}$ (0.096±0.005 mg) littermates (Figure 19).

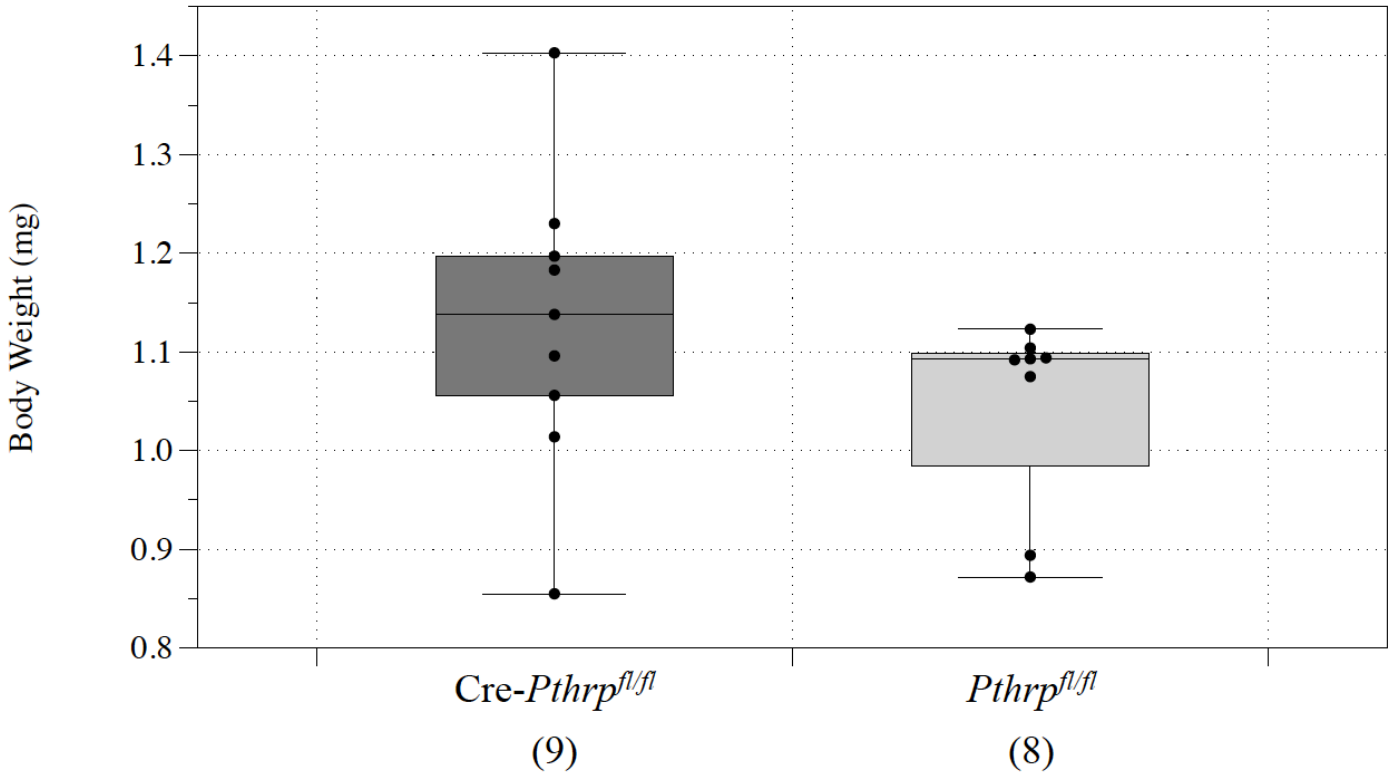

Figure 18: Average body weight of pups at ED 18.5. Fetal body weight did not differ between the fetal genotypes. Statistical analysis was done by student's t-test.

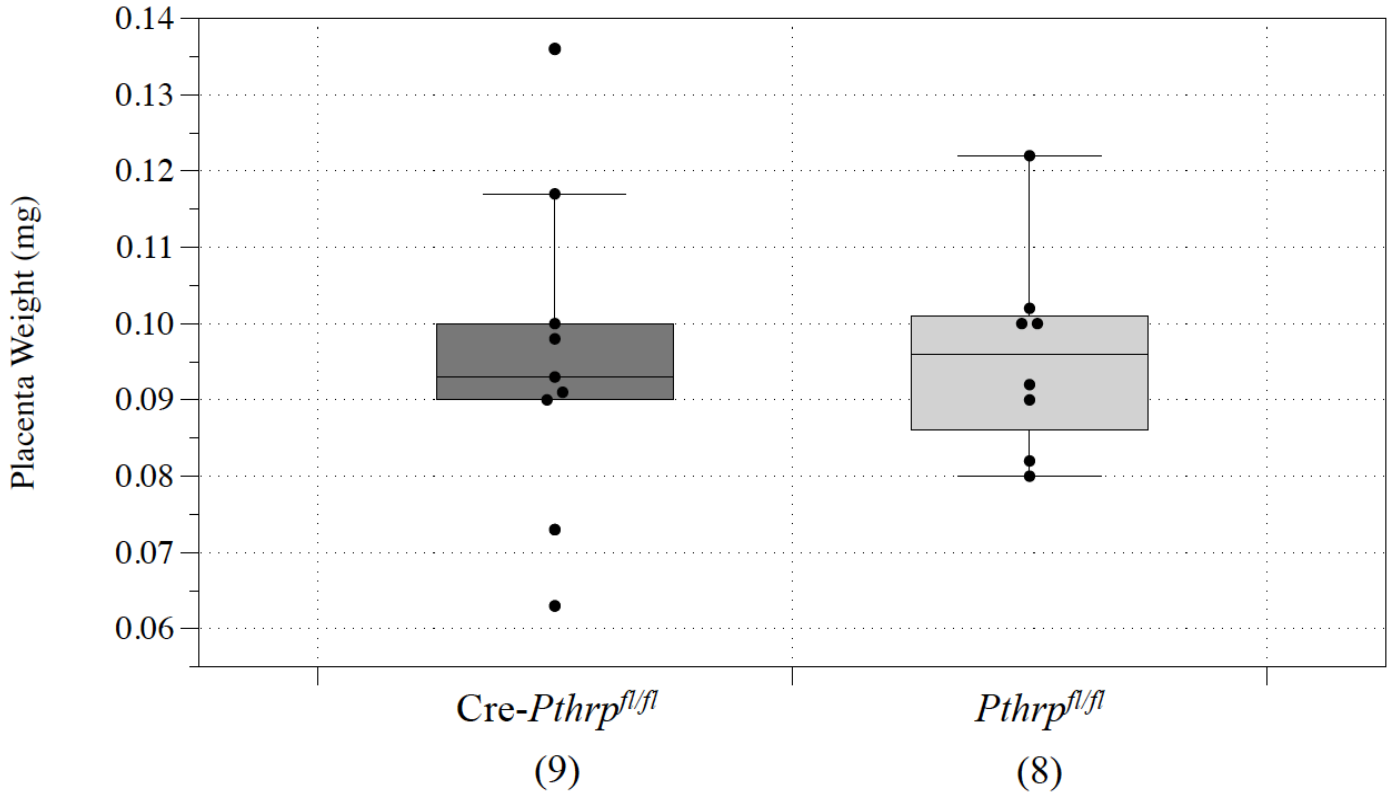

Figure 19: Average placenta weight at ED 18.5. Placenta weight did not differ between fetal genotypes. Statistical analysis was done by student's t-test.

3.2 Serum mineral concentrations

In the global deletion of *Pthrp* the null fetuses experienced hypocalcemia and hyperphosphatemia compared to their littermates. In this model, we wanted to investigate if the absence of PTHrP from the placenta would alter serum calcium and phosphorus concentrations as occurred in the global deletion of PTHrP. For the serum calcium and phosphorus data we did the analysis in two groups. Our primary analysis was on the fetal data comparing the Cre-*Pthrp*$^{fl/fl}$ fetuses to their *Pthrp*$^{fl/fl}$ littermates. Our secondary analysis was comparting the fetal values to the maternal (dam) values.

3.2.1 Calcium

In the primary analysis, the Cre-*Pthrp*$^{fl/fl}$ fetuses showed a decrease in serum calcium, specifically 1.28 ±0.14 mmol/L, compared to their *Pthrp*$^{fl/fl}$, 1.98 ±0.16 mmol/L, $p<0.004$. In the secondary analysis, the Cre-*Pthrp*$^{fl/fl}$ fetuses also showed a significant decrease compared the maternal values, 2.19 ±0.04 mmol/L, $p<0.0001$. While there was no statistically significant difference between the *Pthrp*$^{fl/fl}$ fetuses and the dams.

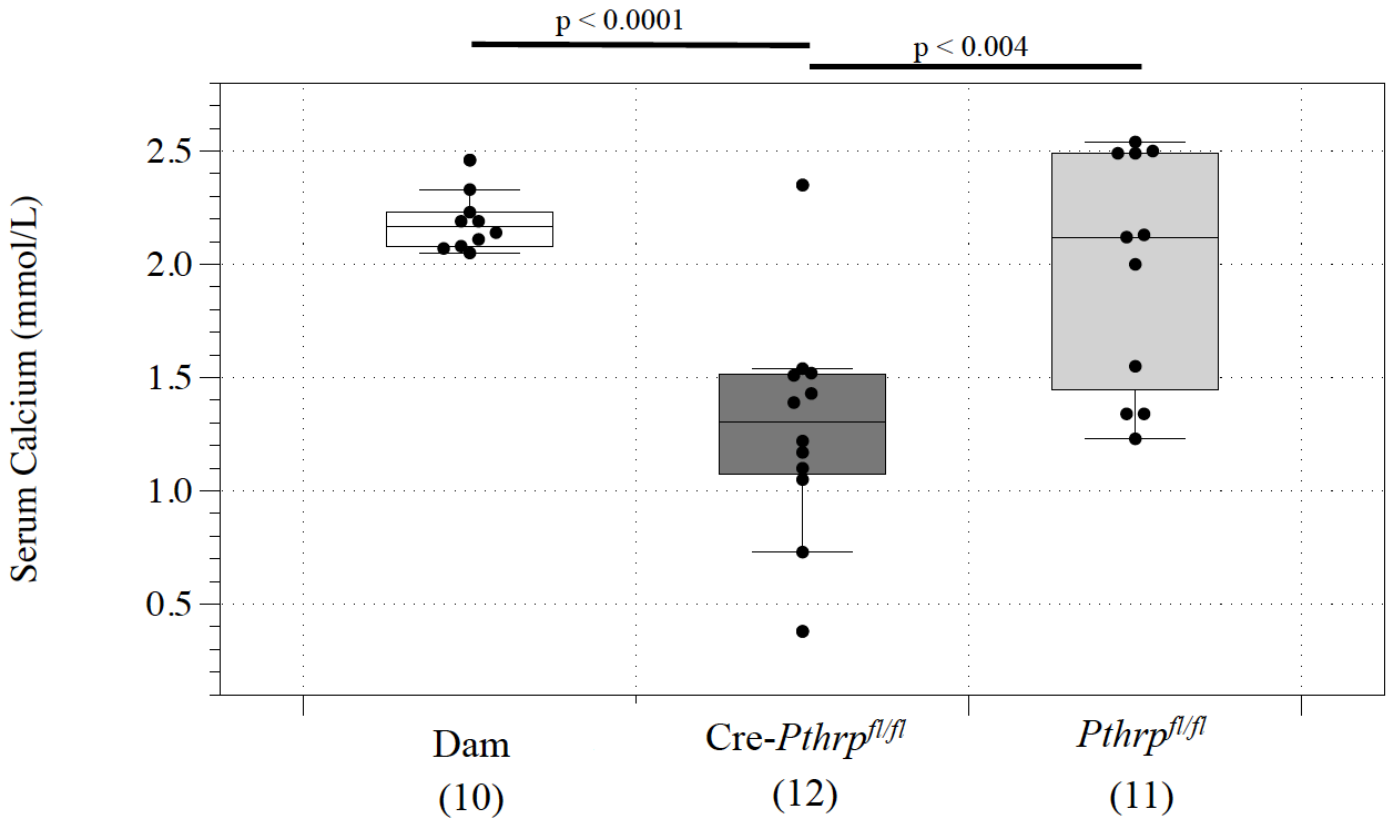

Figure 20: Serum calcium concentration. Cre-*Pthrp*$^{fl/fl}$ fetuses (1.28 ±0.14 mmol/L) displayed hypocalcemia compared to their *Pthrp*$^{fl/fl}$ littermates (1.98 ±0.16 mmol/L, $p<0.004$), and dams (2.19 ±0.04 mmol/L, $p<0.0001$). Statistical analysis was done by one-way ANOVA.

3.2.2 Phosphorus

In the primary analysis, the Cre-*Pthrp*$^{fl/fl}$ fetuses had a significant increase ($p<0.004$) in serum phosphorus concentration, 4.13 ± 0.19 mmol/L, compared to their *Pthrp*$^{fl/fl}$ littermates, 3.26 ± 0.18 mmol/L. In the secondary analysis, both fetal groups had higher serum phosphorus compared to maternal concentration (1.95 ± 0.07 mmol/L, $p<0.0004$).

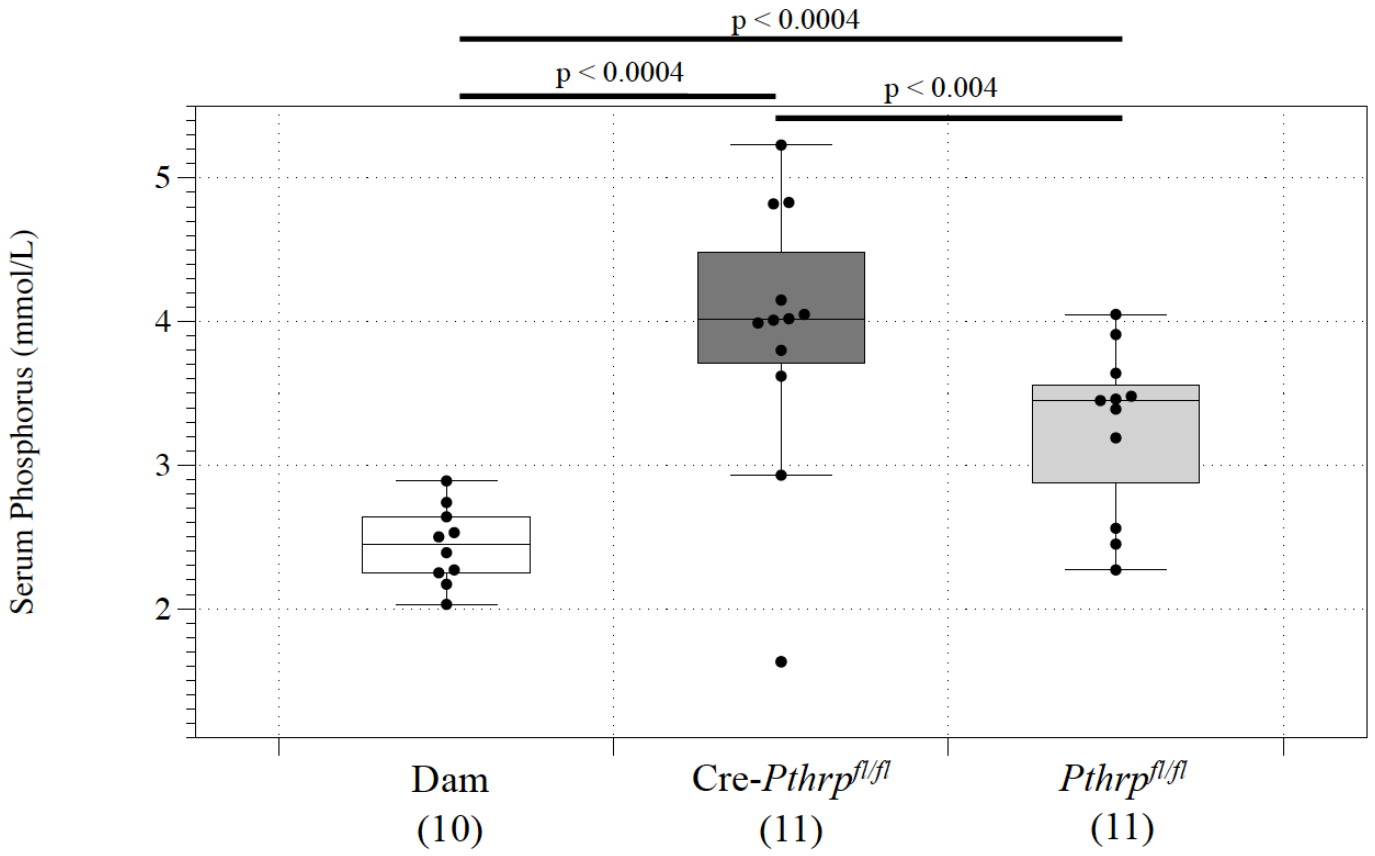

Figure 21: Serum phosphorus concentration. Cre-*Pthrp*$^{fl/fl}$ (4.13 ± 0.19 mmol/L) displayed hyperphosphatemia compared to their *Pthrp*$^{fl/fl}$ littermates (3.26 ±0.18 mmol/L, p<0.004). Both fetal genotypes had higher serum phosphorus compared to the dams, (1.95 ±0.07 mmol/L with p<0.0004). Statistical analysis was done by one-way ANOVA.

3.3 Fetal skeletal ash weight, mineral content, and morphology

Fetal whole bodies were collected on ED 18.5 for fetal skeletal ash weight and content. Fetal tibiae were collected and fixed in formalin on ED 18.5 for skeletal morphology. Fetal ash weight and fetal ash content are indicators of mineralization in the skeleton and differences suggest abnormalities during the mineralization process of fetal development.

There were no differences in fetal ash weight between the fetal genotypes (Figure 22). Skeletal calcium, phosphorus and magnesium content were measured between the fetal genotypes. There were no significant differences between the genotypes for any of these three minerals (Figures 23-25).

Tibiae were then stained with Von Kossa, and counter-stained with methyl green. Von Kossa stains calcium bound to phosphorus, indicating mineralization. We aimed to determine the impact of the putative placental specific PTHrP knockout on the morphology of the chondrocytes as they progress through the zones, the length of the growth plate and the zones, and mineralization pattern shown through relative von Kossa staining of the tibiae to look for any abnormalities. There was no difference in the morphology, length of the growth plates or the zones within it, or mineralization pattern of the tibiae between the fetal genotypes (Figure 26).

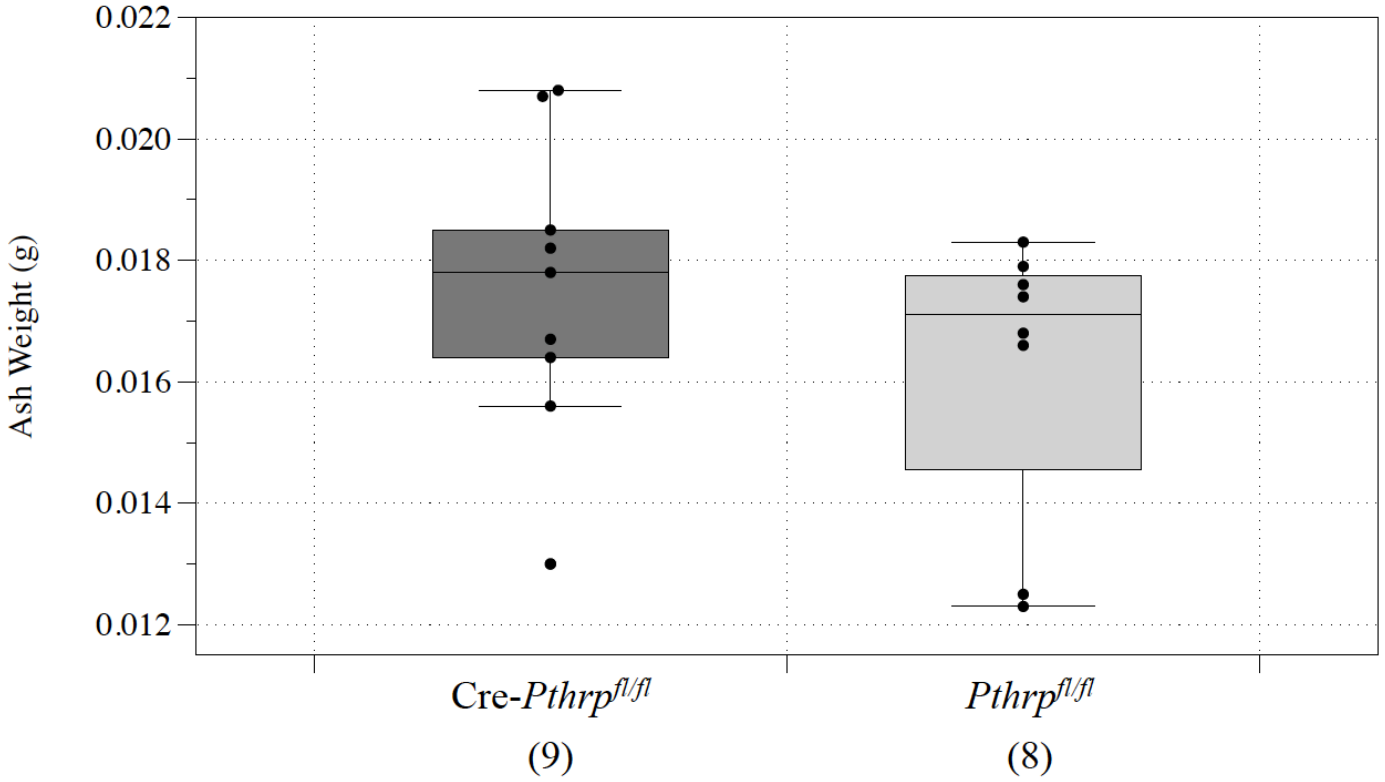

Figure 22: Skeletal ash weight. Mean skeletal ash weight did not differ significantly between the fetal genotypes [Cre-*Pthrp*$^{fl/fl}$ (0.018±0.001 g); *Pthrp*$^{fl/fl}$ (0.016±0.001 g)]. Statistical analysis was done by student's t-test.

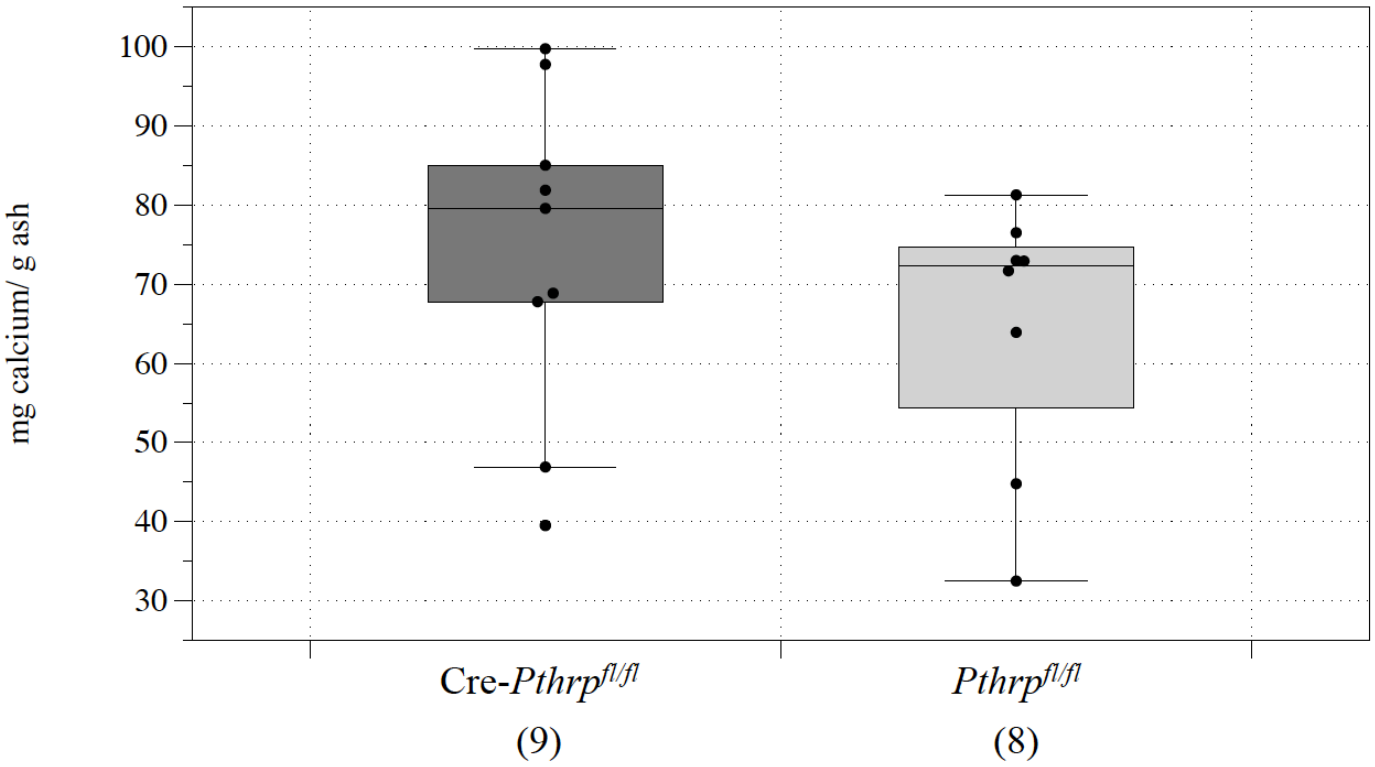

Figure 23: Skeletal ash calcium content. The average skeletal calcium content did not differ significantly between the fetal genotypes [Cre-*Pthrp*$^{fl/fl}$ (74.1±6.9 mg); *Pthrp*$^{fl/fl}$ (64.6±6.0 mg)]. Statistical analysis was done by student's t-test.

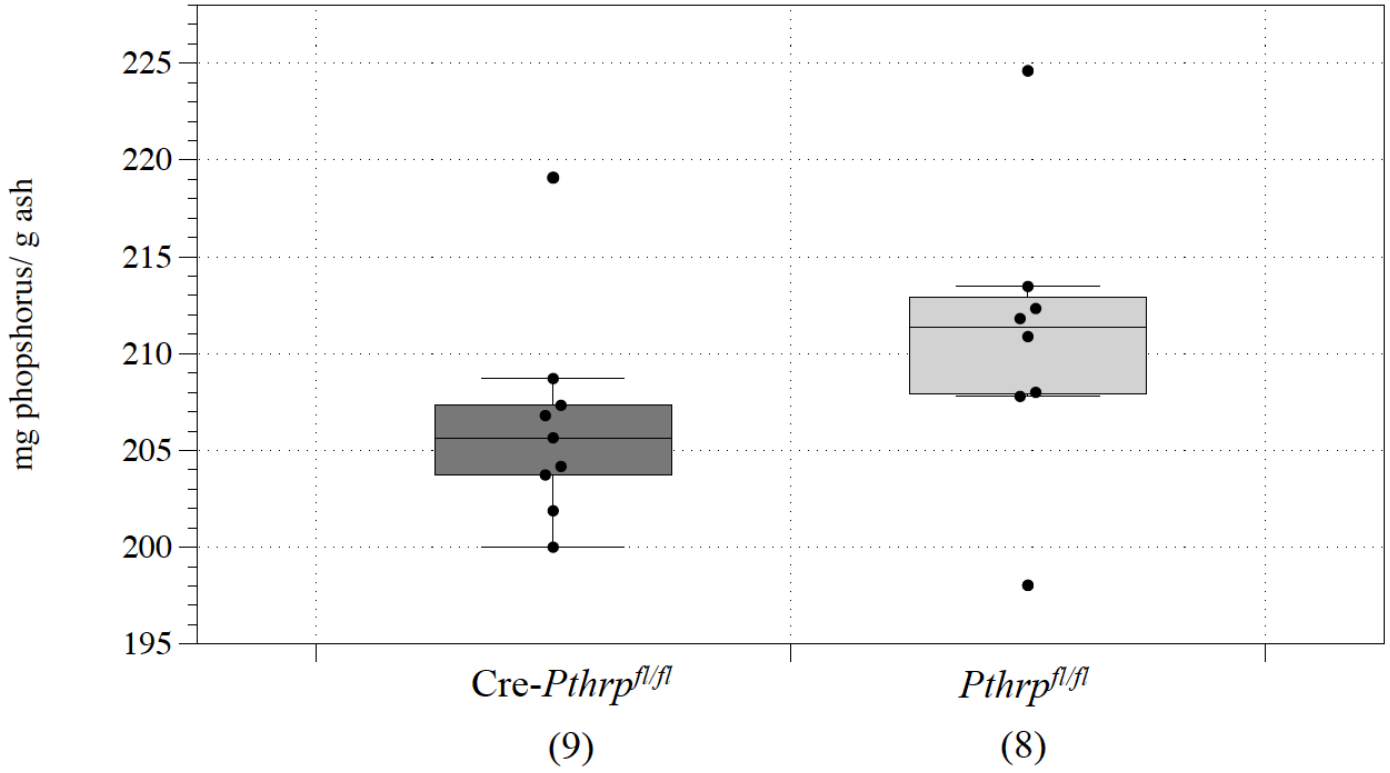

Figure 24: Skeletal ash phosphorus content. The average phosphorus content did not differ significantly between the fetal genotypes [Cre-*Pthrp*$^{fl/fl}$ (206.4±1.8 mg); *Pthrp*$^{fl/fl}$ (210.9±2.6 mg)]. Statistical analysis was done by student's t-test.

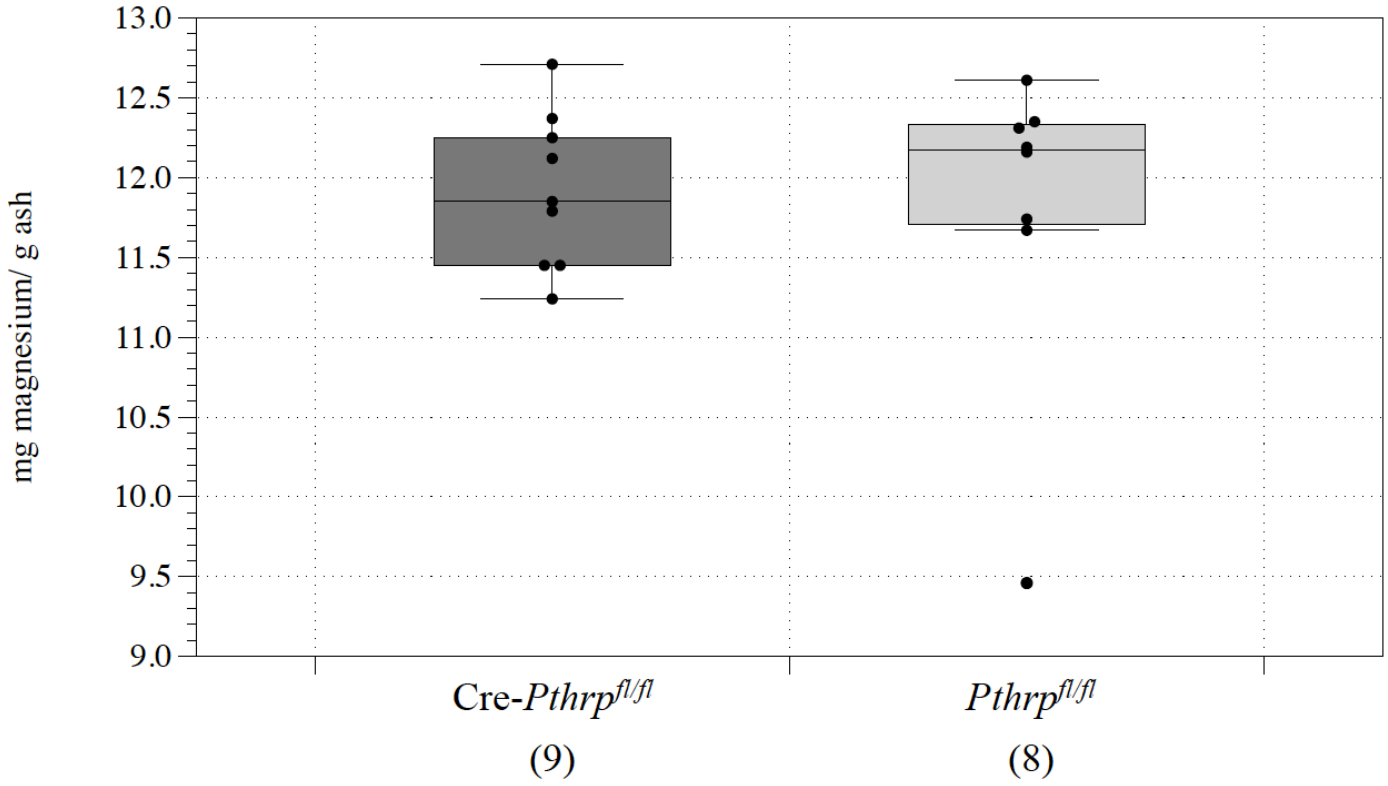

Figure 25: Skeletal ash magnesium content. The average magnesium content did not differ significantly between the fetal genotypes [Cre-*Pthrp*$^{fl/fl}$ (11.9±0.2 mg); *Pthrp*$^{fl/fl}$ (11.8±0.4 mg)]. Statistical analysis was done by student's t-test.

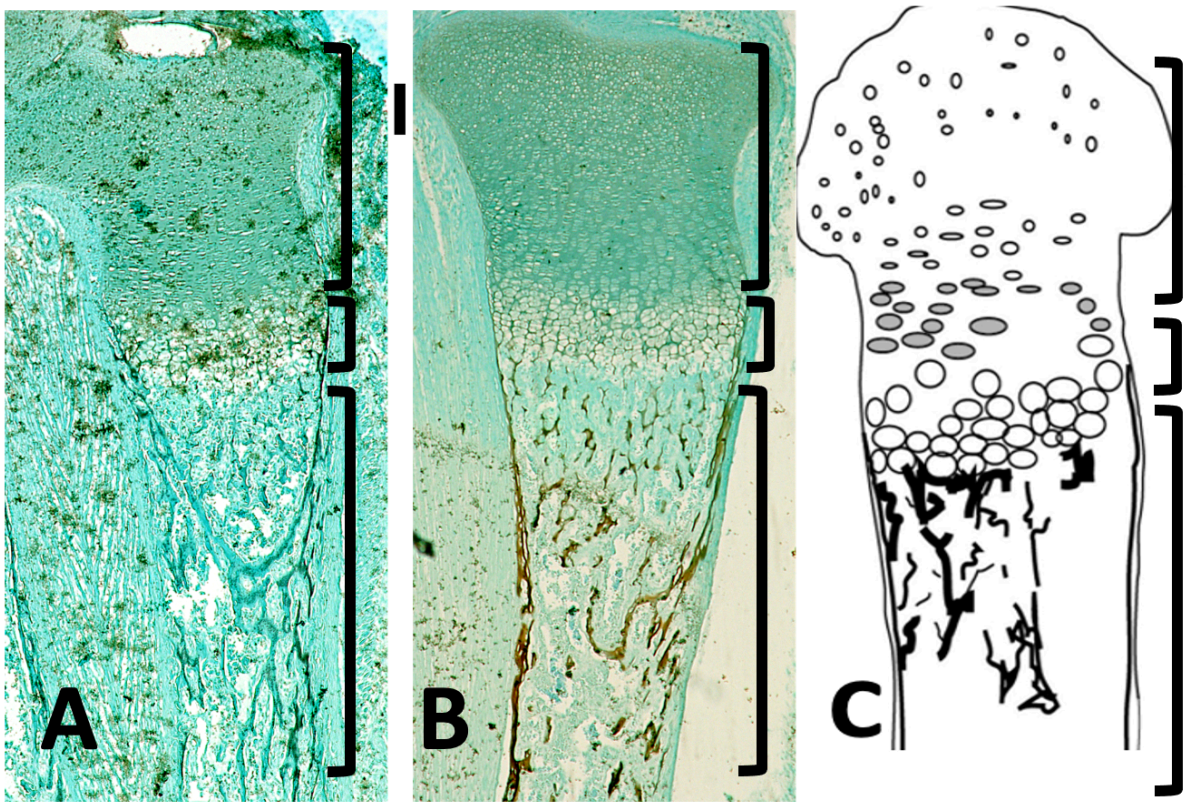

Figure 26: Morphology of fetal tibiae. (A) Cre-*Pthrp*$^{fl/fl}$ (B) *Pthrp*$^{fl/fl}$ (C) Reference photo adapted from Dr. Christopher Kovacs. Used with permission from Elsevier © 2018 (Appendix F).[42] There was no difference in the morphology of Cre-*Pthrp*$^{fl/fl}$ (2) tibiae compared to their *Pthrp*$^{fl/fl}$ (6) littermates, including length, cellular morphology, or mineralization. Scale bar in between (A) and (B) is equal to 100 microns.

3.4 Expression of placental genes as assessed by qPCR

The expression of genes important for calcium and phosphorus homeostasis were measured in the placentas of Cre-*Pthrp*$^{fl/fl}$ fetuses and from their *Pthrp*$^{fl/fl}$ littermates. These genes were chosen because they are involved in the transport of minerals across the placenta. Analysis of specific genes by qPCR showed a modest but significant increase in placental calcium transporter *S100g* in the Cre-*Pthrp*$^{fl/fl}$, 1.33 ± 0.15, compared to *Pthrp*$^{fl/fl}$ fetuses, 1.00 ±0.13, with $p<0.006$. Expression of other genes related to calcium and phosphorus homeostasis including *Pmca1*, *Cyp27b1*, *Cyp24a1*, *Trpv6*, *Pth*, *Vdr*, *Ncx1*, *Pdia3*, and *Pthrp* was unchanged.

Gene	Fold change (Cre-*Pthrp*$^{fl/fl}$ vs. *Pthrp*$^{fl/fl}$)±	P value
S100g	**1.334±**	**< 0.01**
Pmca1	**1.049±**	**NS**
Cyp27b1	**0.647±**	**NS**
Cyp24a1	**1.107±**	**NS**
Trpv6	**0.885±**	**NS**
Pth	**0.331±**	**NS**
Vdr	**1.263±**	**NS**
Ncx1	**1.158±**	**NS**
Pdia3	**1.297±**	**NS**
Pthrp	**1.007±**	**NS**

Table 1: Placental gene expression. qPCR was performed on placentas from Cre-*Pthrp*$^{fl/fl}$ and *Pthrp*$^{fl/fl}$. Placental expression of the gene for calbindin-D-9k (S100g) was increased in the Cre-*Pthrp*$^{fl/fl}$ (1.33 ± 0.15 vs. 1.00 ± 0.13 in WT, $p<0.006$). S100g is a gene involved in placental calcium transport.

IV. DISCUSSION

This study focused on determining the effects of placental-sourced PTHrP and its influence on fetal mineral and bone metabolism in mice. We hypothesized that selective ablation of *Pthrp* from trophoblasts would lead to fetal hypocalcemia, hyperphosphatemia, reduced placental calcium transport, low renal calcium excretion into the amniotic fluid, and reduced skeletal mineral content. To study this, we created a putative conditional knockout of PTHrP in trophoblast cells of fetal mice.

In brief, our main findings from this study revealed that the Cre-*Pthrp*$^{fl/fl}$ fetuses experienced lower serum calcium, higher serum phosphorus, normal skeletal ash weight and ash content, normal tibial morphology, and a slightly increased expression of *S100g* in the placenta compared to their littermates.

4.0 The absence of fetal placental PTHrP induces hypocalcemia and hyperphosphatemia in the Cre-*Pthrp*$^{fl/fl}$ fetuses

The Cre-*Pthrp*$^{fl/fl}$ fetuses had a lower serum calcium concentration compared to that of their *Pthrp*$^{fl/fl}$ littermates and maternal values. This was in keeping with the previous finding of hypocalcemia in the global knockout of PTHrP in mice,[87] except that in our model PTHrP was exclusively deleted from trophoblasts only. In our model, the fetuses had a calcium concentration of 1.28 ±0.14 mmol/L this is consistent with the global PTHrP knockout, the global null fetuses had a calcium concentration of 1.22 ±0.02 mmol/L. The similarity in the values from our placenta knockout and the previously studied global knockout[87] suggests that the hypocalcemia in both models is likely from the loss of PTHrP from the placenta. Hypocalcemia in the Cre-

Pthrp$^{fl/fl}$ fetuses suggests that the placenta is likely the major source of PTHrP influencing calcium homeostasis in the fetus. It may not be the sole source of PTHrP influencing calcium homeostasis, however, the loss of PTHrP from the placenta alone has a large enough impact on fetal calcium homeostasis that its absence will cause a significant decrease of calcium in the serum of the Cre-*Pthrp*$^{fl/fl}$ fetuses. The Cre-*Pthrp*$^{fl/fl}$ fetuses also had a higher serum phosphorus concentration compared to their *Pthrp*$^{fl/fl}$ littermates and maternal values. Once again this is keeping with the previous finding of hyperphosphatemia in the global knockout of PTHrP in mice[87] suggesting that the placenta is the major source of PTHrP influencing phosphorus homeostasis. As our model exclusively deleted PTHrP from the trophoblasts, this finding suggests that the absence of PTHrP from the placenta alone has enough of an impact to increase serum phosphorus in the Cre-*Pthrp*$^{fl/fl}$ fetuses. The hypocalcemia and hyperphosphatemia observed in the Cre-*Pthrp*$^{fl/fl}$ fetuses support our hypothesis, that the placenta is the major source of PTHrP which in turn regulates fetal mineral homeostasis.

As described in fetal pigs and mice, mid-region PTHrP influences serum calcium concentration and placental calcium transport while amino-terminal PTHrP and PTH do not.[13,69,83,87] These findings suggest that PTHrP may be acting an alternative mid-region receptor separate from PTH1R that it shares with PTH.[13,69,83,87] The removal of PTHrP from the placenta influences mineral metabolism as it induced hypocalcemia and hyperphosphatemia in the Cre-*Pthrp*$^{fl/fl}$ fetuses. It is possible that PTHrP from the placenta is circulating in the fetus and acting on the alternative mid-region receptor which is allowing PTHrP to influence systemic mineral concentration during fetal development. This alternative mid-region receptor has not yet been identified but may be a key regulator of PTHrP in fetal mineral homeostasis and bone

development as it is the infusion of mid-region PTHrP which reverses the phenotypes in the animal models and not amino-terminal PTHrP or PTH.[13,69,83,87]

4.1 The absence of fetal placental PTHrP does not change ash weight or ash mineral content in the fetuses

While the serum calcium and phosphorus concentrations were impacted in the absence of placental PTHrP, the Cre-*Pthrp*$^{fl/fl}$ fetuses did not display any changes in ash weight or ash mineral content compared to their *Pthrp*$^{fl/fl}$ littermates. The ash weight is a gross measure of the mineral content of the skeleton, but it is the ash mineral content that gives a more precise measure of the individual mineral content of the ash (the skeleton). In the Cre-*Pthrp*$^{fl/fl}$ fetuses, the ash weight did not differ compared to the *Pthrp*$^{fl/fl}$ littermates. The ash mineral content (which includes calcium, phosphorus, and magnesium) also did not differ among groups. As this model causes ablation of the hormone from only the trophoblasts, PTHrP is still present in other tissues and cell types. Specifically, it is still present in the cells of the skeleton such as the chondrocytes as PTHrP plays a crucial role in the regulation of endochondral ossification.[40,81,85] Therefore, any influence on skeletal development would have been through changes in skeletal mineralization, such as if there was a reduction in the amount of calcium available to be incorporated into the developing skeleton. The hypocalcemia we observed would have predicted lower skeletal mineral content such as in the *Trpv6* null[94], *Hoxa3* null[13], *Pth* null[13], and *Gcm2* null[13] mice. These mice had lower serum calcium concentration with reduced skeletal mineralization and lower ash weight. However, this was not the case with the Cre-*Pthrp*$^{fl/fl}$ fetuses, as they had normal mineralized skeletons and ash weight compared to their *Pthrp*$^{fl/fl}$ littermates. The *Trpv6* null[94] fetuses had a much lower serum calcium levels, 0.89±0.10 mmol/L,

compared to the Cre-*Pthrp*$^{fl/fl}$ fetuses, 1.28 ±0.14 mmol/L. This suggest that while the loss of placental PTHrP does causes hypocalcemia it is not as severe as other models and that the low levels of serum calcium in the Cre-*Pthrp*$^{fl/fl}$ fetuses must not be low enough to detectably impair skeletal mineralization.

4.2 The absence of fetal placental PTHrP does not impact skeletal morphology

Sections of fetal tibiae were stained to look for any abnormalities in the areas where endochondral ossification occurs via the bone cells. The tibial morphology of the Cre-*Pthrp*$^{fl/fl}$ fetuses was normal, with no difference in length or structural organization of the bone collar. Since this is a tissue-specific knockout in the trophoblasts, PTHrP should still be present in chondrocytes. The PTHrP from the chondrocytes will likely be acting locally to control the rate that the cells pass through the zones during endochondral ossification. Specifically, the area of the hypertrophic chondrocytes, which in Figure 26, is the middle section noted in each half of the tibia. This area is the same length in each tibia from the fetal genotypes and the chondrocytes are the same shape and have not stained black from von Kossa, indicating they have not yet mineralized. The normal length and morphology of the hypertrophic zone seen in the tibiae of all the fetal genotypes imply that the normal expression and action of local PTHrP is still occurring. The absence of PTHrP from the placenta does not show the same impact as the global knockout which displayed abnormal and accelerated mineralization of the skeleton.[81] This finding, and the fact that ash weight and content did not differ among the fetal genotypes, indicate that PTHrP from the placenta is not influencing the fetal skeletal development as in its absence the Cre-*Pthrp*$^{fl/fl}$ fetuses do not display abnormal features to their skeleton, likely because the local

PTHrP from the chondrocytes is influencing skeletal development and mineralization as in the global knockout[81] and in mice with overexpression of PTHrP in chondrocytes[85].

4.3 Fetal placental PTHrP increases expression of *S100g*

The Cre-*Pthrp*$^{fl/fl}$ fetuses had an increased expression of *S100g* in the placenta. This finding suggests that PTHrP from the placenta could be influencing placental calcium transport, however this remains to be elucidated. *S100g* encodes for a vitamin D dependent calcium transport (calbindin-D-9k) and an increase of its expression in the Cre-*Pthrp*$^{fl/fl}$ fetuses suggests that hormonal PTHrP is regulating placental calcium transport. However, this is the opposite to what was expected and what was observed in previous PTHrP models where the absence of PTHrP lead to a reduction in the expression of *S100g* in the placenta.[37] This could indicate a compensatory response, in which the increased expression of *S100g* causes an increase in calcium transport to the fetuses in the absence of placental PTHrP to try to stimulate the transport of calcium across the placenta to the developing fetus.. Whether it indicates anything about the rate of calcium transport remains to be seen and will be further discussed in the future directions section of this thesis. Expression of other genes related to calcium and phosphorus homeostasis including *Pmca1, Cyp27b1, Cyp24a1, Trpv6, Pth, Vdr, Ncx1 and Pdia3* were unchanged. These findings could indicate that placental calcium and phosphate transport are not changed.

4.4 Study Strengths and Limitations

4.4.1 Strengths

4.4.1.1 First model of a placental ablation of PTHrP

This study was the first to use the Cre-recombinase system to delete the PTHrP gene

solely in the placenta and study its impact on fetal development. The findings, described in the previous sections, have provided initial evidence for how PTHrP from the trophoblasts impacts fetal mineral homeostasis and bone development and homeostasis. While much remains to be investigated, the results from this project suggest that placental PTHrP may be an important determinant of the fetal serum calcium concentration.

4.4.2 Limitations

4.4.2.1 The knockout model has not been confirmed by qPCR

We have not yet been able to confirm that PTHrP has been successfully ablated from placental trophoblasts. This may relate to known problems with the specificity of primers and antibodies for PTHrP. We used commercial PTHrP Taqman assays with manufacturer's suggested primers, which may not be capturing PTHrP because of its low expression in tissue. Because of this, we cannot be certain that the effects that we are seeing are due to absence of PTHrP in the placenta. This will be discussed in detail in the future direction section 4.6.1 of this thesis as confirming the placental ablation of PTHrP is the next area of focus for the project.

4.4.2.2 Biological sex differences

During the analysis, biological sex of fetuses was not determined through genotyping. Therefore, the data from this project was not analyzed for sex differences. Whether the loss of placental PTHrP will impact one sex more than the other is unknown. This will be an area of focus during the remainder of this project and will be discussed in section 4.6.3.

4.5 Relevance to Human Health

The regulation of bone and mineral homeostasis in adults and children is quite well understood. As described in the introduction, the calciotropic and phosphotropic hormones include PTH, calcitriol, FGF-23, and the sex steroids are responsible for the tight regulation of adult and child bone and mineral homeostasis.[9,13] However, the regulation of fetal bone development and mineral homeostasis are not well understood because (as discussed in the background review) absence of the known calciotropic and phosphotropic hormones have little to no impact on it. While this study suggests that placental PTHrP may regulate fetal serum calcium and phosphorus during murine fetal development, we cannot be certain that this will be the case in humans. By continuing our studies on fetal bone development and mineral homeostasis through animal models we can use the information gained to help predict how these processes occur in human fetuses.

4.6 Future Directions

Creating a putative tissue-specific knockout of *Pthrp* in the fetal trophoblasts it has allowed us to focus on the placenta as a potential source of circulating PTHrP in the fetus. Thus far we observed that Cre-*Pthrp*$^{fl/fl}$ fetuses experienced hypocalcemia, hyperphosphatemia, increased expression in *S100g*, and no change in ash weight, ash mineral content, or skeletal morphology. These findings suggest that placental PTHrP plays a role in regulating systemic calcium concentrations in the fetus. However, there is still much to be investigated before it can be determine whether the placenta is the circulating source of PTHrP in the fetus including confirming the knockout, creating a new mating scheme, and measuring amniotic fluid mineral concentrations and placental calcium transport.

4.6.1 Confirming the placental knockout

During this study, we created a putative placental knockout of PTHrP using the trophoblast specific Ada-Cre and the floxed PTHrP mice. This system has been independently confirmed to work well to ablate other trophoblast genes. Dr.Xia's group provided *in vitro* and *in vivo* evidence that the use of the chimeric Tpbpa/Ada enhancer achieved placental transgene expression but not in maternal organs or in the fetus.[91] Individually, the *Tpbpa* and *Ada* placental enhancers have low expression levels which limits their utility. By developing and characterizing the chimeric enhancer (*Tpbpa/Ada-AdaP*), Dr. Xia's groups reported a high level of trophoblast specific expression in cultured cells and transgenic mice and greater excision of the floxed genes.[91] While the PTHrP mice[90] from Dr.Karaplis' group have been successfully generated with loxP sites which when tested *in vivo* fully replicated the phenotype observed with the global knockout of PTHrP.[81] In our study, we found a phenotype that indicates ablation was achieved, as we have observed hypocalcemia, hyperphosphatemia, and increased *S100g* expression in Cre-*Pthrp*$^{fl/fl}$ fetuses. Typically, we would go on to confirm successful ablation by showing through qPCR that the gene expression has been knocked down, but there was no significant difference in expression of *Pthrp* in the Cre-*Pthrp*$^{fl/fl}$ placentas compared to the *Pthrp*$^{fl/fl}$ placentas. There are a few possibilities to explain why no difference in the gene expression has occurred. First, gene expression for PTHrP has a normal cycle threshold (C_T) value of 30. The C_T value represents the number of amplification cycles it takes to cross the basal threshold level, meaning the lower the C_T value the higher the starting concentration of the gene. A C_T value of 30 means that the expression is low. We are distinguishing low from lower which makes detecting any changes in the expression or its absence extremely difficult. Second, we knocked out PTHrP from only the trophoblasts in the placenta. As described previously, there are other cell types in the placenta

(such as mesenchyme and uterine cells[35]) that would not be subjected to this Cre-directed ablation. Our analysis uses RNA from the entire placenta. Therefore, it is possible that expression in these other cells is interfering with our ability to detect a reduction in *Pthrp* expression within the trophoblasts. Finally, maternal tissue contributes to the placenta but would not be subjected to Cre-directed ablation. Expression of *Pthrp* from such maternal cells could also be interfering with our ability to detect a decrease in placental expression of *Pthrp*. To further confirm the placental ablation model, we will employ two additional methodologies, immunohistochemistry (IHC) and *in situ* hybridization (ISH).

4.6.1.1 IHC

To confirm ablation of PTHrP from the placenta we first want to use IHC, which uses the principle of antibody binding to a specific antigen in biological tissues. We will use commercially available antibodies to check the expression of PTHrP and Ada-Cre in our mice. Most antibodies created for the detection of PTHrP have not been very successful in detecting its expression because of poor specificity and since the expression of PTHrP may be too low. However, in physiological experiments we are currently using a more specific and validated anti-PTHrP antibody (*anti-PTHrP $_{(1\text{-}33)}$ mAbs*)[95] generated by our colleague Dr. Richard Kremer at McGill University. This antibody is highly specific, as it did not react with PTH, and had no cross-reactivity between antibodies and other fragments of PTHrP.[95] Dr. Kremer's group has used this antibody in IHC and so we will do the same using placental sections. The limitation will be whether PTHrP expression is sufficient to detect in the normal versus the ablated placentas. We will use IHC to confirm the presence and absence of the Cre-recombinase in the Cre-*Pthrp$^{fl/fl}$* and *Pthrp$^{fl/fl}$* mice respectively. To do this, we will use placental sections from the

Ada-Cre maintenance colony, the Cre-*Pthrp*$^{fl/fl}$ and *Pthrp*$^{fl/fl}$ fetuses, and we will use placental sections from another colony from our lab as an extra control.

4.6.1.2 ISH

We will use ISH, a technique which locates specific nucleic acid strands in a tissue by using labelled DNA or RNA probes[96], to confirm the presence or absence of PTHrP in the placentas of Cre-*Pthrp*$^{fl/fl}$ and *Pthrp*$^{fl/fl}$ mice respectively. One of the major advantages of using this technique is that it is extremely sensitive, meaning that even at low levels, such as with PTHrP, this method can detect the presence of the mRNA of interest. Furthermore, like IHC, it provides visual localization of the expression, and thus will be able to detect changes in expression within specific cell types, as we have done before using ISH.[37] As with IHC, this technique requires placental sections from the Cre-*Pthrp*$^{fl/fl}$ and *Pthrp*$^{fl/fl}$ fetuses, and we will once again use placentas from another colony from our lab as an extra control group.

4.6.2 New mating scheme to eliminate maternal influences of Ada-Cre

Moving forward, we will also take the mating scheme one step further to eliminate the female breeder from carrying the Cre-recombinase. This is as an extra precaution to eliminate the possibility of knockout of PTHrP influencing the maternal side of the placenta if the Ada-Cre expression is leaky. This cross will be a continuation of the experimental cross that we have previously done with two steps. The first cross takes the *Pthrp*$^{fl/fl}$*; Ada* Cre$^{-/-}$ mice and the Tpbpa/*Ada*-Cre$^{+/+}$ mice to give *Pthrp*$^{fl/+}$*; Ada* Cre$^{+/+}$ offspring, both male and female. The second cross, we took the female *Pthrp*$^{fl/+}$*; Ada* Cre$^{+/+}$ mice and crossed with male *Pthrp*$^{fl/fl}$*; Ada* Cre$^{-/-}$ mice. The litters from this cross were the four fetal genotypes: *Pthrp*$^{fl/fl}$ and *Pthrp*$^{fl/+}$, with half of each

expressing Cre. Following this for the new mating we will take the male *Pthrp*$^{fl/fl}$*; Ada* Cre$^{+/+}$ mice and cross them to the female *Pthrp*$^{fl/fl}$*; Ada* Cre$^{-/-}$ mice. The offspring from this cross will be *Pthrp*$^{fl/fl}$, with half of these expressing Cre (Figure 27). This will remove the dam from carrying the Cre-recombinase and allows for a bigger yield as all the offspring from these will either be the Cre-*Pthrp*$^{fl/fl}$ group or the *Pthrp*$^{fl/fl}$ group.

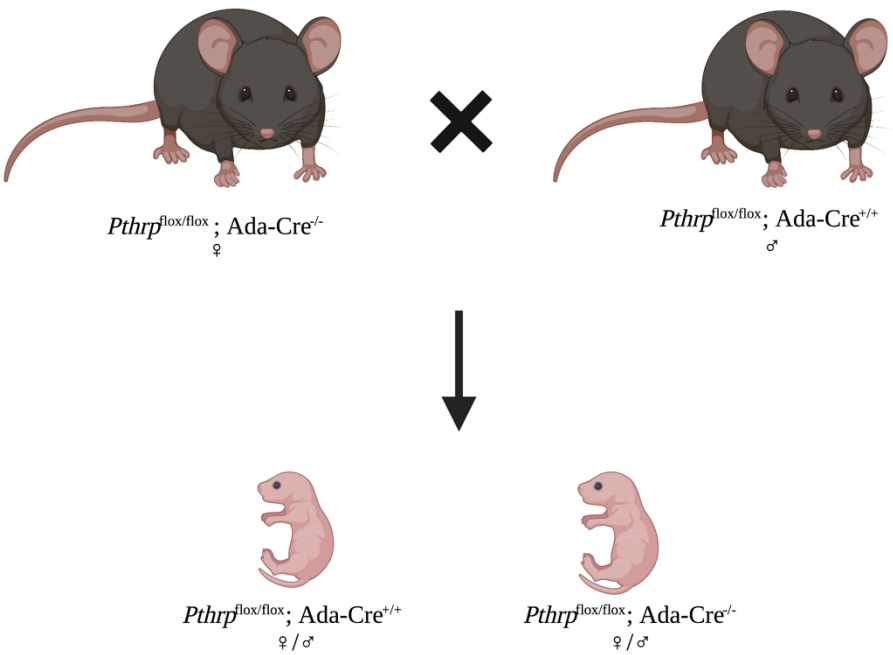

Figure 27: New experimental mating scheme. Male *Pthrp*$^{flox/flox}$*; Ada* Cre$^{+/+}$ mice will be crossed to female *Pthrp*$^{flox/flox}$*; Ada* Cre$^{-/-}$ mice. The females from this cross will euthanized using cervical dislocation at ED 18.5 and pups will be harvested through C-section. The fetal genotypes from this cross will be Cre-*Pthrp*$^{flox/flox}$ and *Pthrp*$^{flox/flox}$. Figure made using www.biorender.com. Permission from BioRender (Appendix O).

4.6.3 Amniotic fluid, placental calcium transport, and biological sex analysis

Finally, we will measure the mineral content of the amniotic fluid and the rate of placental calcium transport in the Cre-*Pthrp*$^{fl/fl}$ fetuses and their littermates. Both measurements will be done at ED 17.5. Amniotic fluid is collected at ED 17.5, a day earlier compared to the other tissue collection, as at ED 18.5 it is much more viscous and the volume decreases. For the collection of amniotic fluid, each fetal sac is pierced, and amniotic fluid is collected into capillary tubes. It is then transferred into individual, labelled, 0.6mL microcentrifuge tubes (ThermoFisher Scientific, Burlington, ON), and stored at -20°C for future analysis. Concentrations of calcium, phosphorus, and magnesium will be measured and compared among the fetal genotypes.

For placental calcium transport, pregnant dams are given intracardiac injection of 50 μCi ^{45}Ca together with 50 μCi ^{51}Cr-EDTA; the latter is passively transferred across the placenta and serves as a blood diffusional marker. Five minutes after the injection, dams are sacrificed using cervical dislocation and a C-section is performed to remove fetuses. Whole fetuses are placed into scintillation vials and solubilized. The ^{45}Ca activity is measured for each fetus using a liquid scintillation counter and the ^{51}Cr activity is measured using a γ-counter. Each fetal ^{45}Ca/^{51}Cr value is normalized within their individual litters to the mean value in the fetuses so that litters can be compared to one another. From this analysis we will be able to determine whether placental calcium transport is impacted in the Cre-*Pthrp*$^{fl/fl}$ fetuses. We expected to see a reduction in the rate of calcium transport across the placentas of the Cre-*Pthrp*$^{fl/fl}$ fetuses as seen in the global[87] and PTH/PTHrP receptor[83] knockouts. However, with the increased expression of *S100g* we may see an increase in placental calcium transport.

Biological sex of the fetuses at ED 18.5 will be determined through genotyping, following the methods described in section 2.1. The results from the collected data will then be analyzed to compare the female pups versus the male pups to determine if the loss of placental PTHrP has different impacts based on biological sex.

4.7 Summary

We hypothesized that placental-derived PTHrP is circulating in the fetus and is responsible for the regulation of mineral homeostasis and placental calcium transport during fetal development. At this point the finding of hypocalcemia and hyperphosphatemia in placental PTHrP null fetuses supports our hypothesis that placental PTHrP is responsible for fetal mineral homeostasis, but additional work is needed to confirm the ablation and fully establish the phenotype. Thus far, it appears that placental PTHrP influences serum calcium and phosphorus concentrations in the fetus but that it does not impact fetal bone development and mineralization. Whether the hypocalcemia and hyperphosphatemia are caused by an alteration in placental mineral transport and renal handling of mineral excretion into the amniotic fluid remains to be determined.

4.8 Conclusion

Cre-*Pthrp*$^{fl/fl}$ fetuses exhibited decreased serum calcium and increased serum phosphorus without changes in skeletal development or mineralization. These findings are consistent with placental PTHrP acting in a hormonal manner to influence the regulation of fetal serum mineral concentrations but while not having a direct influence on skeletal development or mineralization. Whether placental PTHrP influences placental mineral transport remains to be determined.

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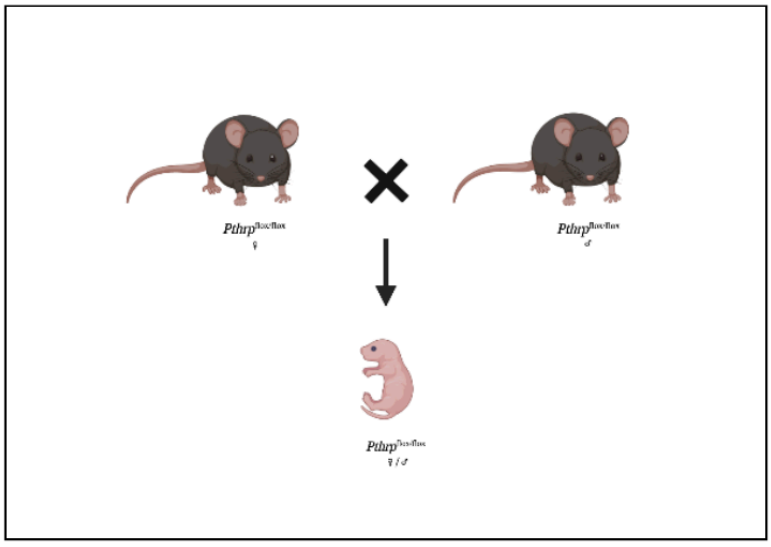

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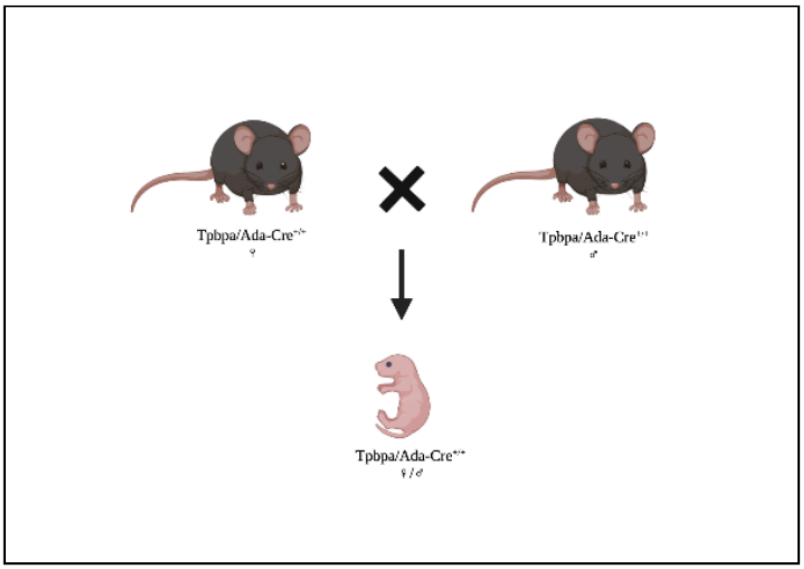

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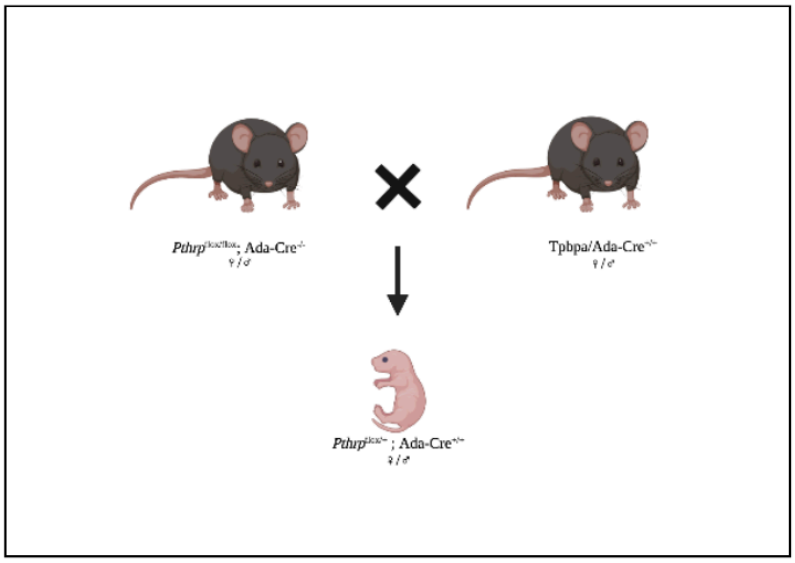

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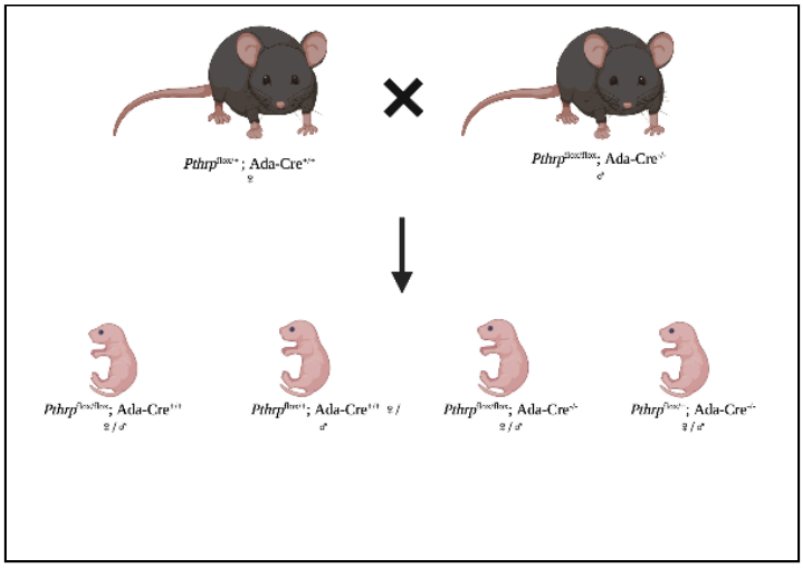

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Appendix M: MUN Animal Care Ethics Approval

From: maw222@mun.ca
Subject: Your Animal Use Protocol Amendment has been Approved Event No. 20220273 - 545238
Date: 9 September2021 at 4:17 PM
To: Kovacs Christopher(Principal Investigator) CKOVACS@mun.ca, Kirby Elizabeth(Co-Investigator) ejkirby@mun.ca
Cc: maw222@mun.ca

Animal Care Committee (ACC)
St. John's, NL, Canada A1C 5S7
Tel: 709 777-6620 acs@mun.ca
https://www.mun.ca/research/about/acs/acc/

Dear: Dr. Christopher Kovacs, Faculty of Medicine\Discipline of Medicine

Researcher Portal File No.: 20220273
Animal Care File:
Entitled: (21-01-CK) Fetal, neonatal and lactational mineral homeostasis in normal and genetically engineered rodents
Status: Active
Related Awards:

Awards File No	Title	Status	
20140593	Regulation of murine calcium and bone metabolism during pregnancy, lactation and post-weaning	Completed	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20150477	University Research Professor 2013	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20191792	Calcitriol-dependent and independent regulation of calcium and bone metabolism during pregnancy, lactation, and fetal/neonatal development	Denied	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
	Adaptations in calcium, phosphate, and bone		1. Research Grant and

20192238	Adaptations in calcium, phosphate, and bone metabolism during pregnancy, lactation, post-weaning, and fetal/neonatal development, using genetically engineered mouse models and observational studies in women	Pending Sponsor Decision	Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20192479	Regulation of murine calcium and bone metabolism during pregnancy, lactation and post-weaning	Completed	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20192741	Adaptations in calcium, phosphate, and bone metabolism during pregnancy, lactation, post-weaning, and fetal/neonatal development, using genetically engineered mouse models and observational studies in women	Denied	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20192980	Calcitriol-dependent and independent regulation of calcium and bone metabolism during pregnancy, lactation, and fetal/neonatal development	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20193093	Material Transfer Agreement 20193093	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20200561	The role of placental parathyroid hormone-related protein (PTHrP) in regulating fetal mineral homeostasis and skeletal development	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20200677	Material Transfer Agreement - Not Publishable	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses

Approval Date: June 05, 2021
Annual Report Due: August 23, 2022
Ethics Clearance Expires: June 05, 2024

Ethics Clearance Expires: June 03, 2024

Your Event [Administrative Amendment] application request to change personnel was considered by the IACC and has been approved.

This ethics clearance includes the following Team Members: Dr. Christopher Kovacs (Principal Investigator)
Mrs. Elizabeth Kirby (Co-Investigator)
Sarah Hartery (Student)
Mr. David Bennin (Student)
Mrs. Brittany Ryan (Student)
Miss Kristi Berit Sellars (Student)
Alexandre Seiji Maekawa (Student)

This ethics clearance includes the following Sponsors: [[[AllSponsorAgencyNames]]
This ethics clearance includes the following related awards:

Awards File No	Title	Status	
20140593	Regulation of murine calcium and bone metabolism during pregnancy, lactation and post-weaning	Completed	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20150477	University Research Professor 2013	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20191792	Calcitriol-dependent and independent regulation of calcium and bone metabolism during pregnancy, lactation, and fetal/neonatal development	Denied	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20192238	Adaptations in calcium, phosphate, and bone metabolism during pregnancy, lactation, post-weaning, and fetal/neonatal development, using genetically engineered mouse models and observational studies in women	Pending Sponsor Decision	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20192479	Regulation of murine calcium and bone metabolism during pregnancy, lactation and post-weaning	Completed	1. Research Grant and Contract Services (RGCS) – St. John's and

			Grenfell Campuses
20192741	Adaptations in calcium, phosphate, and bone metabolism during pregnancy, lactation, post-weaning, and fetal/neonatal development, using genetically engineered mouse models and observational studies in women	Denied	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20192980	Calcitriol-dependent and independent regulation of calcium and bone metabolism during pregnancy, lactation, and fetal/neonatal development	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20193093	Material Transfer Agreement 20193093	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20200561	The role of placental parathyroid hormone-related protein (PTHrP) in regulating fetal mineral homeostasis and skeletal development	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20200677	Material Transfer Agreement - Not Publishable	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses

Amendments will remain active until the protocol expires on June 05, 2024.

Post Approval Exchange (PAX) Requirement
In accordance with the CCAC guidelines, ACC has begun PAX visits for approved protocols. The following criteria are applicable;

1. All category of invasiveness D and E protocols. Category of invasiveness B and C protocols may be included if any of the points below are of concern
2. Protocols with procedures that are of concern to the ACC, which may include invasive or non- invasive procedures
3. Protocols with history of compliance issues
4. Protocols with large animal recovery
5. Protocols with humane endpoints that may be in question

5. Protocols with humane endpoints that may be in question

6. Protocols with procedures that take place in the laboratories rather than in the animal care facilities.

Please be advised that researchers may be contacted for PAX visits in future.

NOTE: You can access a copy of this email at any time under the "Shared Communications" section of the Logs tab of your file in the Memorial Researcher Portal.

Sincerely,

ANULIKA MBAKWE | ACC COORDINATOR
Department of Animal Care Services
Memorial University of Newfoundland
Health Sciences Centre | Room H1848
P: 709-777-6621
E-Mail: ambakwe@mun.ca <grammarly-desktop-integration data-grammarly-shadow-root="true"></grammarly-desktop-integration>

Appendix N: Envigo- Teklad Global 18% Protein Rodent Diet

2018

ENVIGO

Teklad Global 18% Protein Rodent Diet

Product Description- 2018 is a fixed formula, non-autoclavable diet manufactured with high quality ingredients and designed to support gestation, lactation, and growth of rodents. 2018 does not contain alfalfa, thus lowering the occurrence of natural phytoestrogens. Typical isoflavone concentrations (daidzein + genistein aglycone equivalents) range from 150 to 250 mg/kg. Exclusion of alfalfa reduces chlorophyll, improving optical imaging clarity. Absence of animal protein and fish meal minimizes the presence of nitrosamines. **Also available certified (2018C) and irradiated (2918). For autoclavable diet, refer to 2018S (Sterilizable) or 2018SX (Extruded & Sterilizable).**

Ingredients (in descending order of inclusion)- Ground wheat, ground corn, wheat middlings, dehulled soybean meal, corn gluten meal, soybean oil, calcium carbonate, dicalcium phosphate, brewers dried yeast, iodized salt, L-lysine, DL-methionine, choline chloride, kaolin, magnesium oxide, vitamin E acetate, menadione sodium bisulfite complex (source of vitamin K activity), manganous oxide, ferrous sulfate, zinc oxide, niacin, calcium pantothenate, copper sulfate, pyridoxine hydrochloride, riboflavin, thiamin mononitrate, vitamin A acetate, calcium iodate, vitamin B_{12} supplement, folic acid, biotin, vitamin D_3 supplement, cobalt carbonate.

Standard Product Form: Pellet

Macronutrients		
Crude Protein	%	18.6
Fat (ether extract) [a]	%	6.2
Carbohydrate (available) [b]	%	44.2
Crude Fiber	%	3.5
Neutral Detergent Fiber [c]	%	14.7
Ash	%	5.3
Energy Density [d]	kcal/g (kJ/g)	3.1 (13.0)
Calories from Protein	%	24
Calories from Fat	%	18
Calories from Carbohydrate	%	58
Minerals		
Calcium	%	1.0
Phosphorus	%	0.7
Non-Phytate Phosphorus	%	0.4
Sodium	%	0.2
Potassium	%	0.6
Chloride	%	0.4
Magnesium	%	0.2
Zinc	mg/kg	70
Manganese	mg/kg	100
Copper	mg/kg	15
Iodine	mg/kg	6
Iron	mg/kg	200
Selenium	mg/kg	0.23
Amino Acids		
Aspartic Acid	%	1.4
Glutamic Acid	%	3.4
Alanine	%	1.1
Glycine	%	0.8
Threonine	%	0.7
Proline	%	1.6
Serine	%	1.1
Leucine	%	1.8
Isoleucine	%	0.8
Valine	%	0.9
Phenylalanine	%	1.0
Tyrosine	%	0.6
Methionine	%	0.4
Cystine	%	0.3
Lysine	%	0.9
Histidine	%	0.4
Arginine	%	1.0
Tryptophan	%	0.2

Vitamins		
Vitamin A [e, f]	IU/g	15.0
Vitamin D_3 [e, g]	IU/g	1.5
Vitamin E	IU/kg	110
Vitamin K_3 (menadione)	mg/kg	50
Vitamin B_1 (thiamin)	mg/kg	17
Vitamin B_2 (riboflavin)	mg/kg	15
Niacin (nicotinic acid)	mg/kg	70
Vitamin B_6 (pyridoxine)	mg/kg	18
Pantothenic Acid	mg/kg	33
Vitamin B_{12} (cyanocobalamin)	mg/kg	0.08
Biotin	mg/kg	0.40
Folate	mg/kg	4
Choline	mg/kg	1200
Fatty Acids		
C16:0 Palmitic	%	0.7
C18:0 Stearic	%	0.2
C18:1ω9 Oleic	%	1.2
C18:2ω6 Linoleic	%	3.1
C18:3ω3 Linolenic	%	0.3
Total Saturated	%	0.9
Total Monounsaturated	%	1.3
Total Polyunsaturated	%	3.4
Other		
Cholesterol	mg/kg	--

[a] Ether extract is used to measure fat in pelleted diets, while an acid hydrolysis method is required to recover fat in extruded diets. Compared to ether extract, the fat value for acid hydrolysis will be approximately 1% point higher.

[b] Carbohydrate (available) is calculated by subtracting neutral detergent fiber from total carbohydrates.

[c] Neutral detergent fiber is an estimate of insoluble fiber, including cellulose, hemicellulose, and lignin. Crude fiber methodology underestimates total fiber.

[d] Energy density is a calculated estimate of *metabolizable energy* based on the Atwater factors assigning 4 kcal/g to protein, 9 kcal/g to fat, and 4 kcal/g to available carbohydrate.

[e] Indicates added amount but does not account for contribution from other ingredients.

[f] 1 IU vitamin A = 0.3 µg retinol

[g] 1 IU vitamin D = 25 ng cholecalciferol

For nutrients not listed, insufficient data is available to quantify.

Nutrient data represent the best information available, calculated from published values and direct analytical testing of raw materials and finished product. Nutrient values may vary due to the natural variations in the ingredients, analysis, and effects of processing.

Teklad Diets are designed and manufactured for research purposes only.

0915

Teklad Diets + Madison WI + envigo.com + tekladinfo@envigo.com + (800) 483-5523

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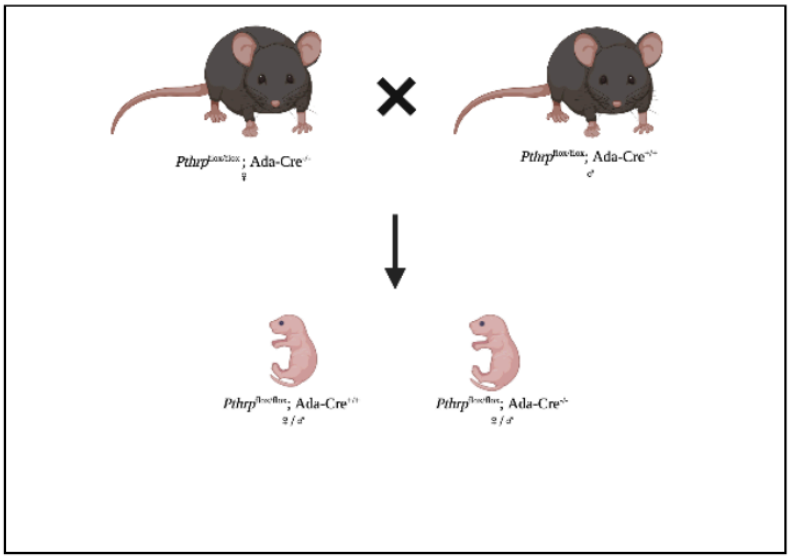

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