CONTRIBUTION OF RANKL REGULATION TO BONE RESORPTION
INDUCED BY PTH RECEPTOR ACTIVATION IN OSTEOCYTES

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ABSTRACT

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PTH increases osteoclasts by upregulating RANKL in cells of the osteoblastic lineage, but the precise differentiation stage of the PTH target cell remains undefined. Recent findings demonstrate that PTH regulates gene expression in osteocytes and that these cells are an important source of RANKL. We therefore investigated whether direct regulation of the RANKL gene by PTH in osteocytes is required to stimulate osteoclastic bone resorption. To address this question, we examined bone resorption and RANKL expression in transgenic mice in which PTH receptor signaling is activated only in osteocytes (DMP1-caPTHR1) crossed with mice lacking the distal control region regulated by PTH in the RANKL gene (DCR−/−). Longitudinal analysis of circulating C-terminal telopeptide (CTX) in male mice showed elevated resorption in growing mice that progressively decreased to plateau at 3-5 month of age. Resorption was significantly higher (~100%) in DMP1-caPTHR1 mice and non-significantly lower (15-30%) in DCR−/− mice, versus wild type littermates (WT) across all ages. CTX in compound DMP1-caPTHR1; DCR−/− mice was similar to DMP1-caPTHR1 mice at 1 and 2 months of age, but by 3 months of age, was significantly lower compared to DMP1-caPTHR1 mice (50% higher than WT), and by 5 months, it was undistinguishable from WT mice. Micro-CT analysis revealed lower tissue material density in the distal femur of DMP1-caPTHR1 mice, indicative of high remodeling, and this effect was partially corrected in compound
mice. The increased resorption exhibited by DMP1-caPTH1 mice was accompanied by elevated RANKL mRNA in bone at 1 and 5 months of age. RANKL expression levels displayed similar patterns to CTX levels in DMP1-caPTH1; DCR⁻/⁻ compound mice at 1 and 5 month of age. The same pattern of expression was observed for M-CSF. We conclude that resorption induced by PTH receptor signaling requires direct regulation of the RANKL gene in osteocytes, but this dependence is age specific. Whereas DCR-independent mechanisms involving gp130 cytokines or vitamin D₃ might operate in the growing skeleton, DCR-dependent, cAMP/PKA/CREB-activated mechanisms mediate resorption induced by PTH receptor signaling in the adult skeleton.

Teresita M. Bellido, PhD, Chair
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LIST OF ABBREVIATIONS

RANKL: Receptor activator of nuclear factor-kB ligand.
M-CSF: Macrophage-colony stimulating factor.
TRAP: Tartrate-resistant alkaline phosphatase.
PTHR1: Parathyroid hormone receptor 1.
OPG: Osteoprotegerin.
DCR: Distal control region.
C-AMP: Cyclic-adenosine monophosphate.
DMP1: Dentin-matrix protein1.
STAT3: Signal transducer and activator of transcription3.
VDR: Vitamin D receptor.
CTX: C-terminal telopeptide.
CHAPTER 1
Introduction

Bone and bone cells

The human skeleton is one of the amazing organs of the human body. The skeleton contributes up to 15 to 20 percent of the total human weight. The skeleton is made of different types of bone: cancellous (trabecular) bone and cortical bone. The skeleton plays an important role in the human body. It provides support and protection to the vital organs like the heart, the lung and the brain. It also contains the bone marrow, where the blood cells are formed (1). Bones are also “a reservoir of calcium, phosphate and other ions that can be released or stored in controlled fashion to maintain constant concentration of these important ions in body fluids” (1). Moreover, the bones contribute in the body movement by increasing the force that is generated by contraction of the skeletal muscle.

Figure 1: Bone cells. The bone has three cell types. Osteoclasts are the bone resorbing cells; osteoblasts are the bone forming cells and Osteocytes are responsible to maintenance bone integrity. Osteoclasts and osteoblasts are located on the bone surface, where osteocytes are embedded within the bone in spaces called lacunae. Osteocytes are the most abundant bone cells (~90-95%).
Bone is a special connective tissue made of three cell types: 1- Osteoblasts, 2- Osteoclasts, and 3- Osteocytes (Figure 1). Each one of these cells has an important function for maintenance of a healthy skeleton.

Osteoclasts are the bone resorbing cells. These cells are important in changing the bone shape, remove old or damaged bone, and resorb unwanted portions of the skeleton to maintain overall bone strength (2). Osteoclasts originate from hematopoietic stem cells (1). Receptor activator of nuclear factor-kB ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) are the two essential factors required for osteoclasts differentiation and growth (2). An increase in RANKL or M-CSF will lead to increased osteoclast differentiation. Osteoclasts are multinucleated cells composed of 4-20 nuclei that attach to the bone surface. These cells are characterized by having a ruffled border and an actin ring that connects them tightly to the bone surface. Secretion of acid by the osteoclast dissolves the bone mineral and secretion of enzymes degrades the protein matrix of the bone. Tartrate-resistant alkaline phosphatase (TRAP) and cathepsin K, produced by osteoclasts, are important enzymes for resorption and their levels in the circulation are indicative of osteoclasts number. After completing their resorption activity, osteoclasts disappear from the bone surface and die by apoptosis (2).

Osteoblasts are the bone forming cells. Osteoblasts are responsible for forming the new bone matrix to replace the old bone. Osteoblasts differentiate from precursors of the mesenchymal lineage. RUNX2 and osterix are transcription factors essential for osteoblast differentiation and without them, there is no mature osteoblasts thus leading
to non-mineralized skeleton (2). Osteoblasts are found on the surface of bone side by side usually in one layer. During bone formation, osteoblasts secrete high amounts of type I collagen and other proteins to form the osteoid. Osteoid is new bone that has not mineralized yet. Then, osteoblasts produce noncollagen proteins such as osteocalcin and alkaline phosphatase to initiate the process of osteoid mineralization (1). Both osteocalcin and alkaline phosphatase can be measured in circulation to determine the activity and the number of osteoblasts in bone. At the end of bone formation, osteoblasts undergo apoptosis, become lining cells or become osteocytes.

Osteocytes are responsible for maintenance of bone integrity. These cells are embedded within the bone in spaces called lacunae. Osteocytes are the most abundant bone cells accounting for up to 90-95% of the total bone cells. Each osteocyte has cytoplasmic dendritic processes that run within canaliculi, thin canals excavated in the mineralized bone. Osteocytes communicate with neighboring cells, cells on the surface, and cells of the bone marrow, via gap junctions and membrane channels that when open allow the passage of chemical messengers (2). In response to both mechanical and hormonal stimuli, osteocytes signal to osteoclasts and osteoblasts to induce changes in bone resorption and formation. Osteocytes are long-lived cells, but they can die prematurely by apoptosis. Local changes in osteocyte apoptosis leads to recruitment of osteoclasts to the vicinity and to initiate resorption that replaces damaged bone, constituting the basis of targeted bone remodeling (2). Recent information has demonstrated that osteocytes also detect changes in the level of hormones, such as estrogen, androgen, glucocorticoids and parathyroid hormone (PTH). Reduction in the
Wnt antagonist sost/sclerostin, expressed by osteocytes, by both loading and activation of the receptor for parathyroid hormone (PTHR), leads to increase Wnt signaling. This increases osteoblast number resulting in enhanced bone formation. PTHR activation in osteocytes also increases expression of osteoclastogenic cytokines and elevated osteoclasts and bone resorption. In the PTHR1 model, the enhanced bone remodeling (resorption and formation) is clearly driven by osteocytes (3-4).

**Bone and parathyroid hormone (PTH)**

PTH is secreted by the chief cells of the parathyroid gland. The main function of PTH is to maintain calcium homeostasis. When calcium levels in blood are low, PTH is secreted to elevate calcium and bring it to normal. In bone, PTH increases osteoclast activity to liberate the calcium stored in the bones. In the kidney, PTH increases calcium reabsorption in the proximal tubule and reduces calcium excreted by the urine. PTH also stimulates the synthesis of 1,25(OH)₂D₃, which is the active form of Vitamin D₃, in the kidney. In turn, 1,25(OH)₂D₃ increases intestinal absorption of calcium. As a result of PTH function calcium levels are maintained within the normal range.

PTH has dual effects on bone. The hormone induces bone resorption (catabolic) and also increase bone formation (anabolic) (5). Bone resorption happens when PTH is elevated in a continuous manner, such as in primary hyperparathyroidism due to benign tumors of the parathyroid gland. By this increase in PTH, osteoclast number increases, leading to exaggerated resorption and bone loss. The increase in PTH increases the expression of RANKL on osteoblastic cells, resulting in more osteoclasts. Furthermore, the increase in PTH stimulates the synthesis of M-CSF and inhibits the expression of
osteoprotegerin (OPG) by osteoblastic cells. On the other hand, if PTH is given intermittently, this leads to more bone formation (5). In this case, PTH works to reduce the amount of osteoblast apoptosis, to increase the osteoprogenitors to be osteoblasts, and to reactivate the lining cells to be osteoblasts (5). All these steps increase the number of osteoblasts and their activity to increase the rate of bone formation.

PTH binds to PTH receptors that are expressed in bone only in cells of the osteoblastic lineage. The PTH receptor is coupled to G-proteins resulting in activation of several downstream signals pathways. In bone, the major effects of PTH can be attributed to cyclic-AMP dependent responses (Figure 2). The activation of the PTHR1 affects bone remodeling and bone formation.

**Figure 2: PTH receptor signaling.** The hormone PTH (red circle) binds to the PTH receptor, which is coupled to G proteins (GPCR), and activates diverse downstream signaling pathways. (6)
Work by Dr. Bellido’s laboratory has demonstrated that “transgenic mice expressing a constitutively active PTH receptor exclusively in osteyocytes exhibit increase bone mass and bone remodeling”, which is a result of increasing the number of osteoblasts and osteoclasts (Figure 3) (3). The increase in osteoblasts is due to reduced sclerostin, increased Wnt signaling and decreased osteoblast apoptosis (3). The increase in osteoclasts results from PTHR1 mediated increase in the production of RANKL and M-CSF. Earlier studies demonstrated that PTH increases the expression of RANKL by acting on a region in the gene called Distant transcriptional Enhancer Region or Distal Control Region (DCR) (Figure 4). The DCR is located at 76kb upstream from the transcriptional start site of the gene (7). Genetically modified mice lacking the DCR do not exhibit an overt skeletal phenotype at birth, but display mild reduction in RANKL in bone, reduced osteoclasts and decreased resorption by 5 months of age.
Figure 4: PTH increases the expression of RANKL by acting on the Distal Control Region (DCR). The image shows all the factors that act to control RANKL expression. We focused on PTH, which activates PTHR1, acts on the DCR region through the activation of protein kinase A (PKA)-cAMP pathway to stimulate RANKL expression (blue rectangle) (8).
Goals of this study

In this study, we hypothesize that increased bone resorption in transgenic mice expressing a constitutively active PTH receptor exclusively in osteocytes (DMP1-caPTHR1) (3) results from direct up regulation of RANKL expression in osteocytes induced by PTH receptor signaling. To test this hypothesis, we crossed the DMP1-caPTHR1 mice with mice in which the promoter of the RANKL gene lacks the DCR, and examined RANKL expression and bone resorption. We found that removal of the DCR of the RANKL gene gradually corrects the increased resorption exhibited by DMP1-caPTHR1, and blunts the high RANKL levels in bone. These findings indicate that osteoclast elevation is due to direct effect of PTH receptor signaling in osteocytes on the RANKL gene.
CHAPTER 2
Materials and Methods

Generating the experimental mice

Experimental animals were generated by crossing transgenic mice expressing a constitutively active PTHR1 in osteocytes (DMP1-caPTHR1) (3) with mice lacking the Distant Transcriptional Enhancer region in the RANKL gen (7) called DCR\(^{\text{WT}}\). Generation of experimental mice was accomplished in two steps. The purpose of step one was to generate DMP1-caPTHR1; DCR\(^{\text{WT}}\) which is a double heterozygous and DCR\(^{\text{WT}}\) which is a heterozygous. So we bred DMP1-caPTHR1 with DCR\(^{\text{WT}}\) and we got these four genotypes and their ratios: 1-DMP1-caPTHR1 (25%), 2-DCR\(^{\text{WT}}\) (25%), 3-DMP1-caPTHR1; DCR\(^{\text{WT}}\) (25%) and 4-WT (25%). In the second step we crossed DMP1-caPTHR1; DCR\(^{\text{WT}}\) with DCR\(^{\text{WT}}\) to obtain experimental animals of four genotypes:
1- DMP1-caPTHR1; DCR\(^{\prime}\) (12.5%), 2- DMP1-caPTHR1 (12.5%), 3- DCR\(^{\prime}\) (12.5%) and 4- WT (12.5%).

All mice were born with a normal size and weight and at the expected Mendelian ratio. After 21 days, these mice were weaned in separate cages for males and females. Experimental mice were fed a regular diet (Harlan/Teklad, Indianapolis, IN, USA) (9) and water (H2O reverse osmosis) ad libitum and maintained on a twelve hours of light and dark cycle (9). Institutional Animal Care and Use Committee at Indiana University School of Medicine approved all the animal protocols for this project. In this project, we used one cohort of male and one cohort of females and each cohort contain 9-16 mice per genotype.
At 8 days of age, pups were tattooed with permanent ink and a 0.5cm piece of tail was obtained to extract DNA. Tissue was dissolved in 75 microliter of alkaline-lysis at 95°C for thirty minutes, and subsequent addition of 75 microliter of neutralizing buffer. DNA lysate was then mixed with appropriate primers (Table 1). The mixture was used for the Polymerase Chain Reaction (PCR), which is a technique used to amplify specific pieces of DNA (10). PCR products were separated by Agarose gel electrophoresis (10). The gel electrophoresis was set for one hours and after that a picture was taken to see the bands and to determine the genotype for each sample by PCR product band size, for example WT= 174bp, DCR= 99bp.

<table>
<thead>
<tr>
<th>GENE</th>
<th>Primer</th>
<th>Sequence</th>
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<td>DCR</td>
<td>dCNS-geno-forward</td>
<td>GGGCTAAAATGAAGGGAGGT</td>
</tr>
<tr>
<td></td>
<td>dCNS-geno-reverse</td>
<td>CCCTCCTCAGTGCTCTCTTG</td>
</tr>
<tr>
<td></td>
<td>dCNS-geno-reverse-2</td>
<td>CCTGTGATTAGGCAGTCCCA</td>
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<tr>
<td>DMP1-caPTHR1 (JANSEN)</td>
<td>JANSEN - forward</td>
<td>CTTTCATTACAGGTAGAGGAAC</td>
</tr>
<tr>
<td></td>
<td>JANSEN - reverse</td>
<td>GCGGTCCCATCGCCAC</td>
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</tbody>
</table>

Table 1: Sequence of Primers used for genotyping experimental mice.

Bone mineral density (BMD)

Bone mineral density was measured using a PIXIImus densitometer (GE Medical System, Madison, WI, USA), which is a DXA imaging using two x-ray beams with different energy levels (11). Experimental mice from 1 month to 5 months were anesthetized by inhalation of 2.5% isoflurane (VEDCO, INC, St. Joseph, MO, USA) mixed with oxygen (1.5 litter/minute) (9) to immobilize them during the scan. BMD, BMC and bone area for the
whole body (excluding the head and tail), the femur and the lumbar spine were quantified.

**Micro-computed tomography (Micro-CT)**

Another method we used to study the bone phenotype was Micro-CT. We analyzed left femurs of males at 5 months of age (n=5) from each of the genotypes outlined above. All bones were cleaned and muscles and the soft tissues were removed. Bones were stored in 70% ethanol at 4°C until the day of the scan. Before the scan, the bones were wrapped in para-film to keep them moist and then scanned by Micro-CT (Skyscan 1172, Skyscan, Kontich, Belgium), at 6-micron resolution. Data were reconstructed after the scan to be in 3D representation, and reconstructed images of the mid-shaft and the distal femur were analyzed.

**Quantification of circulating resorption and bone formation markers**

Plasma was collected from the experimental mice at one, two, three, four and five months of age to measure C-telopeptide of collagen type 1a (CTX) (9). CTX was measured by using a enzyme-linked immunosorbent assay to quantify and determine the bone related degradation products resulting from the resorption activity of osteoclasts (Ratlaps™ EIA, Immunodiagnostic Systems, Fountain Hills, AZ, USA). Alkaline Phosphatase, which is a bone formation marker, was measured using the AMP Buffer (Randox # AP 3802) method on a Randox Daytona analyzer (Randox Laboratories Limited, Crumlin, Country Antrim, United Kingdom).
Gene expression analysis by Quantitative PCR

Calvaria bones from 5 month old DMP1-caP<sup>+/WT</sup>; DCR<sup>−/−</sup>, DMP1-caPTH<sub>1</sub><sup>+/WT</sup>, WT and DCR<sup>−/−</sup> mice were snap frozen at sacrifice. Total RNA was purified from bone using Ultraspec reagent (Biotecx Laboratories, Houston, TX, USA) according to the manufacturer’s instructions. Gene expression data was analyzed by quantitative PCR as previously described (3) using primer probe sets from Applied Biosystems (Foster City, CA, USA) or from Roche Applied Science (Indianapolis, IN, USA) (Table 2). “Relative mRNA expression levels were normalized to the housekeeping gene ribosomal protein S2 using the ΔCt method.” (9)

<table>
<thead>
<tr>
<th>GENE</th>
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<tr>
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<td>Forward- Primer</td>
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<tr>
<td></td>
<td>Reverse- Primer</td>
<td>CGTGGTACCAAGAGGACAGAGT</td>
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<td></td>
<td>Probe#</td>
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<tr>
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Table 2: Primers that were used for gene expression and their sequence.

Statistical analysis

Data from our study were analyzed using SigmaStat (SPSS Science, Chicago, IL, USA). All data are presented as the mean ± standard deviation (SD). The statistical differences between groups were evaluated using student’s t-test. P value less than 0.05 was accepted as significant.
Chapter 3

Results

To determine whether bone resorption induced by activation of PTH receptor signaling in osteocytes is due to regulation of the RANKL gene, we generated mice expressing a constitutively active of PTHR1 especially in osteocytes lacking the DCR (DMP1-caPTH1; DCR<sup>−/−</sup>). Longitudinal bone mineral density (BMD) measurements were taken at 1, 2, 3, 4 and 5 months of age for separate cohorts of female and male mice. Plasma samples were collected from all mice at every time point to measure CTX and alkaline phosphatase. Cohorts of male and female mice were sacrificed at 1 month and 5 month of age to collect bones for gene expression, Micro-CT and histomorphometric analysis.

**DCR removal gradually corrects the high resorption exhibited by DMP1-caPTH1 mice**

Longitudinal analysis of CTX for male mice showed elevated resorption in all genotypes at the first two months of age and started to decrease at 3-5 months (Figure 5A). Resorption was significantly elevated about 100% in DMP1-caPTH1 mice compared to WT mice at all ages. The analysis showed a non-significant decrease in resorption (15-30%) in DCR<sup>−/−</sup> compared with WT mice also at all ages. CTX levels in compound DMP1-caPTH1; DCR<sup>−/−</sup> mice were not significantly different from DMP1-caPTH1 mice at 1 and 2 months of age. However, the high resorption exhibited by DMP1-caPTH1 mice was gradually corrected in the DMP1-caPTH1; DCR<sup>−/−</sup> mice. Thus, at 3 months resorption in DMP1-caPTH1; DCR<sup>−/−</sup> mice was significantly lower compared to DMP1-caPTH1 mice (50% over WT). This significant decrease continued until CTX
levels in DMP1-caPTHR1; DCR<sup>−/−</sup> mice became comparable to WT levels at 5 months of age (Figure 5).

Figure 5: The increased resorption exhibited by DMP1-caPTHR1 mice was corrected in DMP1-caPTHR1; DCR<sup>−/−</sup> male mice. CTX measured in plasma from 1 month to 5 month for DMP1-caPTHR1, with and without DCR. A) Longitudinal analysis of CTX for the male cohort from 1 to 5 month. Symbols represent the means ± SD. B) CTX levels at 1 month and 5 month. Bars represent means ± SD. N=7-16 mice per group. *<i>p</i> < 0.05 DMP1-caPTHR1 versus DMP1-caPTHR1; DCR<sup>−/−</sup>, #<i>p</i> < 0.05.
Similar results were found in the longitudinal analysis of CTX for females. Resorption was elevated at the first two months for all genotypes (Figure 6A). Similar to males, resorption was about 70-100% higher over WT littermates in DMP1-caPTHR1 at all ages and it showed a non-significant decrease (7-13%) in DCR\textsuperscript{−/−} mice compared to WT at all ages. DMP1-caPTHR1; DCR\textsuperscript{−/−} female mice had similar CTX levels to DMP1-caPTHR mice for the first two months. But at 3 months DMP1-caPTHR1; DCR\textsuperscript{−/−} female mice showed a significant decrease in CTX compared to DMP1-caPTHR1 mice. This decrease was maintained at 4 and 5 months of age but, in contrast to male mice, it never reached WT levels (Figure 6). Thus, in female mice, the absence of DCR didn’t correct completely the high resorption induced by the DMP1-caPTHR1 transgene.
RANKL and M-CSF expression is reduced in bone of DMP1-caPTHR1; DCR^-/- mice

We next determined whether the DCR deletion in the DMP1-caPTHR mice altered RANKL mRNA expression in bone. RANKL mRNA levels were increased in bones from DMP1-caPTHR1 mice at 1 and 5 months, consistent with high resorption. Moreover, RANKL closely matched resorption in DMP1-caPTHR1; DCR^-/- mice as its levels were similar to DMP1-caPTHR1 mice at 1 month and reduced to WT levels at 5 month of age. M-CSF levels were also high in DMP1-caPTHR1 mice at 1 and 5 months compared with WT. M-CSF elevation in DMP1-caPTHR1 mice was significantly decreased in DMP1-caPTHR1; DCR^-/- mice at 5 months (Figure 8).

Figure 6: The increased resorption exhibited by DMP1-caPTHR1 mice was reduced in DMP1-caPTHR1; DCR^-/- female mice. CTX measured in plasma from 1 month to 5 month for DMP1-caPTHR1, with and without DCR. A) Longitudinal analysis of CTX for the female cohort from 1 to 5 month, Symbols represent means ± SD. B) CTX levels at 1 month and 5 month for male cohort. Bars represent the means ± SD. N=6-18 mice per group. *p <0.05 DMP1-caPTHR1 versus DMP1-caPTHR1; DCR^-/-, #p <0.05.

Figure 7: The increased resorption exhibited by DMP1-caPTHR1 mice was reduced in DMP1-caPTHR1; DCR^-/- female mice. CTX measured in plasma from 1 month to 5 month for DMP1-caPTHR1, with and without DCR. A) Longitudinal analysis of CTX for the female cohort from 1 to 5 month, Symbols represent means ± SD. B) CTX levels at 1 month and 5 month for male cohort. Bars represent the means ± SD. N=6-18 mice per group. *p <0.05 DMP1-caPTHR1 versus DMP1-caPTHR1; DCR^-/-, #p <0.05.
**Figure 7:** RANKL expression is reduced in bone of adult DMP1-caPTHR1; DCR/− male mice. qPCR analysis of RANKL mRNA in calvaria from 1 month and 5 month old male mice for all genotypes. Bars represent means ± SD of 3-5 mice per group. #p <0.05 WT versus DMP1-caPTHR1 mice.

**Figure 8:** M-CSF Expression is significantly reduced in adult DMP1-caPTHR1; DCR/− male mice. qPCR analysis of M-CSF mRNA in calvaria from 1 month and 5 month old male mice for all genotypes. Bars represent means ± SD of 3-5 mice per group. #p <0.05 WT versus DMP1-caPTHR1; *p <.05 DMP1-caPTHR1 versus DMP1-caPTHR1; DCR/− mice.
High bone remodeling in DMP1-caPTHR1 mice was partially corrected in DMP1-caPTHR1; DCR\textsuperscript{−/−} mice

Micro-CT analysis of the distal femur of 5 month old mice showed significant decrease in material density in DMP1-caPTHR1 mice compared with WT littermates, which indicates high bone remodeling resulting in less mineralized bone (Figure 9). Reduction in material density was partially corrected in DMP1-caPTHR1; DCR\textsuperscript{−/−} mice, consistent with decreased rate of bone remodeling leading to better mineralized bone.

Alkaline phosphatase measured in serum of 5 months old male mice was elevated in DMP1-caPTHR1 mice compared to WT mice (Figure 10). However, this increase was markedly reduced in DMP1-caPTHR1; DCR\textsuperscript{−/−} mice.

**Figure 9:** The increased bone remodeling in DMP1-caPTHR1 is partially corrected by the removal of DCR. Bone material density was determined by micro-CT analysis of distal femur from 5 month old male in all groups. Bars represent the means ± SD of 5-7 mice per group. \#p < 0.05.

**Figure 10:** The high bone formation exhibited by DMP1-caPTHR1; DCR\textsuperscript{−/−} mice was reduced by removing the DCR from the RANKL gene. Alkaline phosphatase was measured in serum from 5 month old male mice for all groups. Bars represent means ± SD of 7-11 mice per group. \#p < 0.05.
Longitudinal analysis of Bone Mineral Density (BMD) showed no effect of removal the DCR.

Longitudinal BMD analysis revealed no significant changes in total, femoral, and spinal BMD in DMP1-caPTHR1; DCR<sup>−/−</sup> compared to DMP1-caPTHR1 littermates male or female (Figure 11). However, spinal BMD showed a trend to increase not significant in DMP1-caPTHR1; DCR<sup>−/−</sup> mice compared to DMP1-caPTHR1 mice at 3 and 4 months of age for males and at 2 and 3 months of age for females (Figure 11). This finding is consistent with previous evidence demonstrating that the main effect of absence of the DCR is to inhibit resorption in cancellous bone. The lack of significant effect of DCR deletion on BMD might be due to the combined overall decreased resorption and decreased formation, as evidenced by decreased alkaline phosphatase (Figure 10).
Figure 11: BMD analysis shows no effect of removal of the DCR for both male and female cohorts. Longitudinal analysis of Femoral, Total and Spinal BMD in both males and females at 1 month intervals up to 5 months of age. Symbols are means of 7-19 mice/group ± SD. A) Longitudinal BMD analysis for male littermates for all groups. B) Longitudinal BMD analysis for female littermates for all groups. The lines in the spine BMD indicates a trend of increase not significant in DMP1-caPTHr1; DCR compared to DMP1-caPTHr1 mice.
Chapter 4

Discussion

It has been long recognized that PTH increases osteoclasts by upregulating RANKL in osteoblastic cells, but the precise differentiation stage of the PTH target cell remains undefined. Recent findings demonstrate that PTH regulates gene expression in osteocytes and that these cells are an important source of RANKL. The objective of this work was to establish whether direct regulation of the RANKL gene by PTH in osteocytes is required to stimulate bone resorption. The data demonstrate that resorption induced by PTH receptor signaling requires direct regulation of the RANKL gene in osteocytes in skeletally mature mice (Figure 12). Thus, whereas DCR-independent mechanisms involving gp130 cytokines or vitamin D₃ might operate in the growing skeleton, DCR-dependent, cAMP/PKA/CREB-activated mechanisms mediate resorption induced by PTH receptor signaling in the adult skeleton. CTX measurement for both male and female cohorts showed no significant changes between DMP1-caPTHR1 and

Figure 12: Resorption controlled by DCR in mature skeleton. PTHR1 signaling through c-AMP acts directly on the DCR to control RANKL expression and resorption in mature skeleton. The question mark indicates uncertainty of the source of RANKL that increases the resorption in young skeleton. The increased resorption in the young skeleton could come from gp130 cytokines through STAT3, from $1,25(OH)_2D_3$ through VDR, or from both.
DMP1-caPTHR1; DCR\(^{-/-}\) mice in the first two months. Moreover, RANKL expression was increased in DMP1-caPTHR1; DCR\(^{-/-}\) 1 month old mice. This may indicate that there are other factors different from the DCR that affect RANKL expression in the growing skeleton. Osteocyte-derived cytokines of the gp130 family, such as interleukin (IL)-6, IL-11 or oncostain M (OSM) could be involved in the stimulation of RANKL, since PTH increases gp130 in bone marrow cells (8).

However, at 5 months, CTX showed a significant reduction in DMP1-caPTHR1; DCR\(^{-/-}\) compared with DMP1-caPTHR1 mice. This reduction proves that the DCR (cAMP) dependent mechanisms have a direct effect on RANKL expression when stimulated by the active PTH receptor in osteocytes. Gene expression showed a reduced level of basal RANKL mRNA in DMP1-caPTHR1; DCR\(^{-/-}\) adult mice (5 month old), which is comparable to WT. This evidence further supports that DCR has a major role in controlling the expression of RANKL, therefore control resorption, in skeletally mature animals.

RANKL and M-CSF are two major factors that contribute to osteoclast differentiation and maturation. In the present study, we found that M-CSF mRNA levels exhibited similar pattern to RANKL mRNA levels at both 1 and 5 month of age. Thus, at 1 month of age RANKL and M-CSF were increased and at 5 month of age RANKL and M-CSF expression was reduced in DMP1-caPTHR1; DCR\(^{-/-}\) mice. Deletion of the DCR removes the ability of PTHR/cAMP pathway to increase only RANKL expression. Thus, our results demonstrate that M-CSF regulation is secondary to RANKL regulation. Our findings are the first to demonstrate this regulation in vivo and are consistent with an earlier study in vitro showing that RANKL augmented the production of M-CSF (12).
Therefore, when RANKL is elevated, M-CSF expression will be elevated resulting in more osteoclasts, more bone resorption and higher bone remodeling rate.

DMP1-caPTHR1; DCR⁻/⁻ mice showed reduced levels of the bone formation marker alkaline phosphatase in the circulation compared to DMP1-caPTHR1 mice. Because the primary effect of the DCR removal is to decrease RANKL expression and resorption, the decrease in alkaline phosphatase is likely due to coupling, that is “normal coordination between bone resorption and bone formation” (13). This means that when bone resorption is reduced, there will be a reduction in bone formation and vice versa. Many mechanisms could contribute to coupling between bone resorption and bone formation. It has been proposed that the release of some factors from the bone matrix as a result of osteoclast activity, such as TGFβ, could lead to recruitment of osteoblast progenitors and stimulation of their differentiation (13). Therefore, osteoblast maturation depends on osteoclast activity in the bone matrix, so when osteoclast number is reduced that leads to reduction in osteoblast number and bone formation rate. Additionally, mature osteoclasts might produce factors that increase recruitment of osteoblast precursors, promote their survival and increase their differentiation, such as sphingosine 1-phosphate (S1P) and BMP6 (14).

DMP1-caPTHR1 mice exhibit elevated bone remodeling as well as bone formation (3). The increase in bone formation is due suppression of osteocyte specific product sclerostin/sost and increase Wnt signaling. Wnt signaling works to increase osteoblast differentiation and reduce osteoblast apoptosis. All these factors lead to increase bone formation. The increased bone remodeling can be explained by increasing
RANKL and M-CSF which promote osteoclastogenesis. We then had expected that by inhibiting resorption in combination DMP1-caPTHR1; DCR\textsuperscript{-/-} mice, bone mass will increase even more due to the combination of increasing bone formation dependent on Wnt signaling and decreasing resorption dependent on the DCR. However, we found no significant effect of removing the DCR over the DMP1-caPTHR1 alone. We think that this is due to simultaneous reduction in bone formation, as indicated by lower alkaline phosphatase in the compound mice. Further studies will address directly this hypothesis by measuring bone formation rate and osteoblast number by histomorphometry in DMP1-caPTHR1; DCR\textsuperscript{-/-} mice and their cohort littermates.

In conclusion, the present study demonstrates that the constitutively active PTHR1 expressed exclusively in osteocytes acts directly on the DCR region to promote RANKL expression. The control of RANKL expression has a major impact on differentiation and survival of osteoclasts, which determine the rate of remodeling. Understanding the pathways that control RANKL expression will help design better therapeutic approaches to inhibit bone loss.
Reference List


