ROLE OF IGF-I AS A POTENTIAL MEDIATOR FOR THE SKELETAL EFFECTS OF BONE ANABOLIC AGENTS

By

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To Cary, Rodrigo and Ricardo
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<th>Full Form</th>
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<tbody>
<tr>
<td>ALS</td>
<td>acid-labile subunit</td>
</tr>
<tr>
<td>AN</td>
<td>anorexia nervosa</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ASBMR</td>
<td>American Society for Bone and Mineral Research</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AVMA</td>
<td>American Veterinary Medical Association</td>
</tr>
<tr>
<td>BFR</td>
<td>bone formation rate</td>
</tr>
<tr>
<td>BGFLAP</td>
<td>bone gamma carboxyglutamic acid-containing protein</td>
</tr>
<tr>
<td>BMC</td>
<td>bone mineral content</td>
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<tr>
<td>BMD</td>
<td>bone mineral density</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
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<tr>
<td>BMUs</td>
<td>basic multicellular units</td>
</tr>
<tr>
<td>BS</td>
<td>bone surface</td>
</tr>
<tr>
<td>BV</td>
<td>bone volume</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CKD</td>
<td>chronic kidney disease</td>
</tr>
<tr>
<td>COX-2</td>
<td>cyclooxygenase-2</td>
</tr>
<tr>
<td>Ct</td>
<td>cycle threshold</td>
</tr>
<tr>
<td>DAN</td>
<td>differential screening-selected gene aberrant neuroblastoma</td>
</tr>
<tr>
<td>DKK1</td>
<td>Dickkopf 1</td>
</tr>
<tr>
<td>DXA</td>
<td>dual x-energy x-ray absorptiometry</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immune sorbent assay</td>
</tr>
<tr>
<td>EP</td>
<td>receptors for E series of prostaglandins</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>GH</td>
<td>growth hormone</td>
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<tr>
<td>GHRH</td>
<td>growth hormone-releasing hormone</td>
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<tr>
<td>GPCRs</td>
<td>G-protein-coupled receptors</td>
</tr>
<tr>
<td>HBM</td>
<td>high bone mass syndrome</td>
</tr>
<tr>
<td>HET</td>
<td>heterozygous</td>
</tr>
<tr>
<td>hPTH</td>
<td>human parathyroid hormone</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>IEMA</td>
<td>immunoenzymometric assay</td>
</tr>
<tr>
<td>IGFBP</td>
<td>insulin-like growth factor binding proteins</td>
</tr>
<tr>
<td>IGF-I</td>
<td>insulin-like growth factor I</td>
</tr>
<tr>
<td>IRS-1</td>
<td>insulin receptor substrate-1</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>LID</td>
<td>liver deficient</td>
</tr>
<tr>
<td>LRP</td>
<td>lipoprotein receptor-related protein</td>
</tr>
<tr>
<td>MAR</td>
<td>mineral apposition rate</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage colony stimulating factor</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MS</td>
<td>mineralizing surface</td>
</tr>
<tr>
<td>NAMS</td>
<td>North American Menopause Society</td>
</tr>
<tr>
<td>NHANES</td>
<td>National Health and Nutrition Examination Survey</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>NOF</td>
<td>National Osteoporosis Foundation</td>
</tr>
<tr>
<td>Ob.S</td>
<td>osteoblast surface</td>
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<tr>
<td>Oc.S</td>
<td>osteoclast surface</td>
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</table>
ODF      osteoclast differentiation factor
OPG      osteoprotegerin
OPGL     osteoprotegerin ligand
OPPS     osteoporosis-pseudoglioma syndrome
OS       osteoid surface
Osx      osterix
PCR      polymerase chain reaction
PGE\textsubscript{2} prostaglandin E\textsubscript{2}
PGs      prostaglandins
pQCT     peripheral quantitative computerized tomography
PRL      prolactin
PTH      parathyroid hormone
PTHrP    PTH-related protein
qPCR     quantitative polymerase chain reaction
RANK     receptor activator of nuclear kappa\(\beta\)
RANKL    receptor activator of nuclear kappa\(\beta\) ligand
rhGH     recombinant human GH
rhIGF-I  recombinant human IGF-I
RIN      RNA integrity number
RT-PCR   reverse transcriptase polymerase chain reaction
Runx 2   runt-related transcriptional factor 2
SEM      scanning electron microscopy
SOST     sclerostin gene
Tb.N     trabecular number
TB.Sp    trabecular separation
Tb.Wi    trabecular width
TGFβ    transforming growth factor beta
TH    thyroid hormone
TIDM    type I diabetes mellitus
TNF    tumor necrosis factor
TRANCE    tumor necrosis factor-related activation-induced cytokine
TSH    thyroid stimulating hormone
VEH    vehicle solution
Wnt    wingless/mammalian homologue of drosophila gene wingless
Growth hormone (GH) has a critical role in the regulation of longitudinal bone growth, skeletal maturation, and maintenance of adult bone mass. Insulin-like growth factor I (IGF-I) is considered the prime mediator for the skeletal effects of GH.

Parathyroid hormone (PTH) has potent anabolic effects and its use as an osteoporosis therapy is approved by the Food and Drug Administration. Prostaglandin E₂ (PGE₂) can also induce cortical and trabecular bone formation in animal models. The role of IGF-I as a potential mediator for the bone anabolic effects of PTH and PGE₂ is controversial as in vivo and in vitro studies have yielded conflicting results.

The objectives of this study were: 1- Evaluate the dwarf rat (dw'/dw') as an animal model for the effects of GH and IGF-I deficiency on the skeleton, as related to clinical conditions in which serum levels of IGF-I are decreased but not abolished; 2- Compare the skeletal effects of PTH and PGE₂ treatment in dwarf rats and their background strain, Lewis rats; 3- Determine the expression of genes related to bone formation and resorption. At 9 weeks of age, female Lewis and dwarf rats were treated daily for 2 weeks with vehicle, hPTH 1-34 at a dose of 50 µg/kg body weight, or PGE₂ at a dose of 3 mg/kg body weight (N=7-10/group). Serum IGF-I was measured by ELISA, and bone
changes were analyzed by histomorphometry, peripheral quantitative computerized tomography (pQCT), and biomechanical testing. RNA was isolated from bone tissue for evaluation of gene expression by RT-PCR.

Dwarf rats exhibited markedly lower IGF-I serum levels, and decreased bone mass, strength, and formation compared to Lewis rats. PTH and PGE\textsubscript{2} treatment increased bone mass and formation in both dwarf and Lewis rats. Sclerostin, a potent negative regulator of bone formation, was downregulated in PTH- and PGE\textsubscript{2}-treated dwarf rats, suggesting that sclerostin inhibition could be related to the persistent bone anabolic effects of PTH and PGE\textsubscript{2}, despite low IGF-I serum levels in dwarf rats. Therefore, under the conditions of this study, normal serum levels of IGF-I are not essential for the bone anabolic effects of PTH and PGE\textsubscript{2}.
Osteoporosis and Osteopenic Conditions

Osteoporosis is a systemic skeletal disease characterized by low bone mineral density (BMD), deterioration of bone tissue with disruption of bone architecture, compromised bone strength and an increase in the risk of fracture. It affects primarily the elderly, and, in particular women, representing a major public health problem. It is responsible for an estimated 90% of all hip and spine fractures in white American women ages 65 to 84, according to the 2010 evidence-based position statement published by The North American Menopause Society (NAMS, 2010).

Based on data from the National Health and Nutrition Examination NOF Survey III (NHANES III), the NOF (National Osteoporosis Foundation) has estimated that more than 10 million Americans have osteoporosis and an additional 33.6 million have low bone density of the hip, with related fractures having an estimated cost of $17 billion in 2005 (NOF, 2008).

Furthermore, osteopenia, or low bone mineral density, is not only found in postmenopausal women, but also in other estrogen deficient bone loss conditions, such as women with anorexia nervosa and hypothalamic amenorrhea, as well as in the juvenile onset of osteoporosis (Grinspoon et al., 1999, Giustina et al., 2008, Hofman et al., 2009). Oncological patients under treatment must also be considered, as chemotherapy adversely affects bone metabolism and osteoblast activity (Davies et al., 2002, Hurson et al., 2007). Glucocorticoid-induced osteoporosis (GIOP) is another significant problem, as these potent anti-inflammatory drugs can cause rapid bone loss and increase the risk of fractures (Hofbauer et al., 1999, Kream et al., 2008).
Osteopenia and/or osteoporosis can also be seen in renal osteodystrophy, as a consequence of an extra skeletal systemic disorder of mineral and bone metabolism, which occurs in patients with chronic kidney disease (CKD) (Martin and Gonzalez, 2007, Kalantar-Zadeh et al., 2010). Alcohol abuse, immobilization, reduced physical activity, and low dietary calcium levels mainly during periods of rapid bone growth, all can lead to a decrease in bone mass and osteopenia, with a predisposition to fractures. These conditions can sometimes be assessed, in clinical situations, through bone biopsy and histomorphometric analysis, that are powerful and informative diagnostic tools for the determination of bone abnormalities. More commonly, non-invasive methods, such as bone imaging and serum biomarkers for bone formation and resorption, are used to diagnose osteoporosis and monitor the effectiveness of treatments.

Bone, despite its static appearance, is a dynamic tissue going through constant modeling and remodeling throughout life. Bone modeling, an uncoupled process of bone formation and resorption, occurs mostly during skeletal growth, and it serves mainly to maintain bone shape and mass. Remodeling is a coordinated process in which osteoclasts resorb bone, followed by bone formation through osteoblast activity. Growth hormone (GH) and insulin-like growth factor I (IGF-I) are essential for longitudinal bone growth and can potentially influence the regulation of bone modeling and remodeling as well (Isaksson et al., 1982, Isgaard et al., 1986, Ohlsson et al., 1998, Ohlsson et al., 2009). Depression of the GH/IGF-I axis has been commonly associated with disturbances in bone metabolism and mass, and is consequently related to many osteopenic conditions in humans and in animal models.
**Growth Hormone (GH) and Insulin-Like Growth Factor I (IGF-I)**

Growth hormone (GH) plays an important role in the regulation of longitudinal bone growth, and also in the metabolism and maintenance of adult bone mass. GH or somatotropin is an anabolic peptide hormone released from the somatotrophs, cells in the anterior pituitary gland. GH secretion occurs under the main regulation of GH-releasing hormone (GHRH) and somatostatin, the two main hypothalamic regulators of GH, which exert stimulatory and inhibitory influences, respectively. GH is released in a pulsatile mode, under circadian cycle influence, being secreted mainly at night, during sleep. There is an influence of sex steroids as well, and puberty has a great effect on the amplitude of GH releasing pulses, with both estrogens and androgens stimulating higher pulses (Jansson et al., 1985). GH secretion can also be stimulated by ghrelin, a peptide expressed mainly in the stomach, but also found in other organs, such as the kidney, pancreas, hypothalamus and pituitary (Molina, 2006a). In addition, the secretion of GH can be altered by glucocorticoids, when administered over prolonged periods of time, leading to osteoporosis, as they inhibit the secretion of GH in response to GHRH in healthy subjects (Giustina et al., 1995).

There are other hormones, such as thyroid hormone (TH), and hypothalamic peptides and neurotransmitters, that can affect the regulation of GHRH and somatostatin release, and consequently, GH synthesis (Molina, 2006a). Most importantly, GH release is also inhibited by insulin-like growth factor I (IGF-I), produced mainly in the liver, in a classic negative feedback mechanism of hormone control (Ohlsson et al., 1998, Rosen, 2008). According to the classical concept of the “Somatomedin Hypothesis”, proposed by Salmon and Daughaday about fifty years ago, GH indirectly stimulates IGF-I (or somatomedin-C, as initially denominated) synthesis
and secretion by the liver, which acts as a mediator of the growth and metabolic effects of GH (Salmon and Burkhalter, 1997, Daughaday, 2000). GH plays an important role in the regulation of longitudinal bone growth, and also in the metabolism and maintenance of adult bone mass, acting directly on GH receptors in bone cells and indirectly stimulating IGF-I synthesis by the liver.

IGF-I is an important anabolic growth factor for bone, and is considered the prime mediator of the skeletal effects of GH (Ohlsson et al., 1998, Giustina et al., 2008, Ohlsson et al., 2009). Discovered 50 years ago, it was first described as a soluble factor induced by GH that had insulin-like properties and acted on body growth (Rosen and Niu, 2008). IGF-I is a small 7 kDa peptide that still presents new questions about its metabolic effects and action pathways in several tissues, with special emphasis on bone growth and bone formation (Rajaram et al., 1997, Rosen, 2008).

The highest expression of IGF-I occurs in the liver, where it is primarily secreted and transported to other tissues to act as an endocrine hormone (Ohlsson et al., 2009). Outside the liver, bone is the richest source of IGF-I and IGF-II in mammalian organisms (Rosen and Niu, 2008). It is also synthesized by a variety of other tissues, exerting then tissue-specific paracrine and autocrine effects. In bone tissue, IGF-I is synthesized by many cells, including cells of the osteoblastic lineage, as well as chondroblasts and osteoclasts (Molina, 2006a, Bou-Gharios and Crombrugghe, 2008). IGF-I can stimulate bone formation, protein synthesis, increases replication of cells of the osteoblastic lineage, enhances fibroblast proliferation, increases type I collagen production by osteoblasts, enhances matrix apposition rates, and decreases collagen

A group of six insulin-like binding proteins (IGFBP-1 to -6) regulate the biological actions of IGF-I in circulation and tissue, constituting a complex system for regulating IGF-I activity. IGFBPs can both have stimulatory and/or inhibitory effects on IGF-I function. Binding of IGFBPs to IGF-I usually impedes the interaction of IGF-I to its receptor (IGFR), and therefore inhibits IGF-I actions. On the other hand, binding to IGFBPs also avoids the proteolytic degradation of IGFs by specific serine proteases, increasing their bioavailability in local tissues. In this manner, IGFBPS have a central position in the bioavailability and distribution of IGFs in the extracellular environment (Kelley et al., 1996). The predominant or most abundant form is IGFBP-3, which is the main binding protein in serum, carrying IGF-I in a ternary complex consisting of one molecule of IGF-I, one molecule of IGFBP-3 and one molecule of a protein named acid-labile subunit (ALS). The association of these three proteins forms a complex that prolongs the half-life of the IGFs in the circulation, acting like a reservoir in the organism. Other IGFBPs can bind to IGF-I, forming binary complexes able to cross the capillary barrier and achieve selective transport to different tissues. IGFBPs are produced in several tissues involving intricate regulatory mechanisms (Molina, 2006a). IGFBP-3 is also the predominant binding protein in bone, and hepatic IGFBP-3 and synthesis of ALS are under GH stimulation (Rosen and Niu, 2008).

Low circulating levels of IGF-I are linked to low bone mineral density (BMD), predisposing to the development of osteoporosis and a greater risk of fractures (Niu and Rosen, 2005). In fact, both GH secretion amplitudes and IGF-I and IGFBP-3 serum
levels decrease with age, as the GH/IGF-I axis undergoes natural changes over the life span. Several of these changes in the GH/IGF-I axis have been observed in patients with osteoporosis, as a correlation occurs between low serum IGF-I and IGFBP-3 levels in females with postmenopausal osteoporosis and the likelihood of hip and spinal fractures (Sugimoto et al., 1997). Patients with anorexia nervosa, a condition characterized by low body weight and amenorrhea, also show a markedly decreased BMD, with a high propensity for fractures, and resistance to GH and estrogen treatment. In fact, there is a two- to three-fold increase in fractures in patients with anorexia, associated with low levels of IGF-I, osteopenia and abnormal bone microarchitecture (Lawson et al., 2010, Jacobson-Dickman and Misra, 2010). IGF-I circulating levels are also dramatically reduced in patients with diabetic bone disease. In children with new onset of type I diabetes (TIDM) a 73 % fall in IGF-I serum levels was observed accompanying the downregulation of hepatic IGF-I release (Bereket et al., 1995). Chronic immobilization, as well as space flight, leads to bone loss due to inhibited bone formation. It was proposed that skeletal unloading interferes with the proliferation of osteoblasts and their precursors by inhibiting IGF-I signaling pathways (Sakata et al., 2004).

GH has been successfully used to treat children with growth impairment, resulting in increased skeletal growth. Regarding therapeutic applications, there is one indication of treatment with IGF-I approved in the United States, for Laron syndrome, in which patients lack the GH receptor (GHR) and present very low levels of IGF-I, but high levels of GH. Despite this very specific condition, recombinant human IGF-I (rhIGF-I) and recombinant human GH (rhGH) are not usually indicated for the treatment of
osteoporosis or other osteopenic conditions. Nonetheless, GH/IGF-I axis depression is associated with many of the disorders involving bone loss, and the influence of low GH and IGF-I levels on the action of the drugs routinely used must be better investigated. Studying these growth factors, deeply involved in bone metabolism, is of great importance to improve the available armamentarium of drugs used in the treatment of osteoporosis. The most commonly used drugs, such as estrogen, raloxifene, biphosphonates, and calcitonin, inhibit osteoclast-mediated bone loss, but are unable to stimulate bone formation and restore bone mass to normal levels.

**Parathyroid Hormone**

Parathyroid hormone (PTH) has this targeted bone anabolic capability, and is currently approved by the FDA for the treatment of osteoporotic patients, but it is used only for high fracture risk patients, as it requires daily injections. Interestingly, PTH was considered, at first, only as a primarily catabolic hormone, stimulating bone resorption and the release of calcium into the circulation in hypocalcemic conditions, in order to re-establish calcium homeostasis. Yet, in the kidney, PTH promotes calcium reabsorption and inorganic phosphorus excretion in the urine. Through indirect actions on the gastrointestinal tract, PTH also leads to greater calcium absorption, stimulating the hydroxylation of 25-hydroxyvitamin D₃, at the 1-position, leading to the active form 1,25-dihydroxyvitamin D, or calcitriol. Even very small changes in calcium serum levels can induce the parathyroid glands to increase remarkably PTH secretion. The anabolic effects of PTH were initially noticed in animal studies using parathyroid gland extract, about 80 years ago, and when hPTH started to be pharmacologically synthesized, studies about the potential bone anabolic actions of PTH were undertaken. After initial skepticism about the paradoxical actions of PTH, being both catabolic and anabolic, it
was accepted that continuous high serum levels or doses lead to catabolic effects, while intermittent low doses are associated with anabolic effects on bone (Kousteni and Bilezikian, 2008). Several studies extensively demonstrated the anabolic effects of PTH on the skeleton, increasing bone formation and remodeling, increasing bone density and improving the quality of skeletal microarchitecture (Dempster et al., 1993, Finkelstein et al., 1994, Gunness, 1995, Jilka, 2007, Canalis et al., 2007).

Mechanisms for PTH to induce bone formation include stimulation of growth factors, especially IGF-I. Transient treatment of rat calvarial cell cultures with PTH stimulated collagen synthesis by osteoblasts, but IGF-I neutralizing antibodies prevented this effect (Canalis et al., 1989). Gene expression for IGF-I was also found to be upregulated in bone cell cultures and in bone tissue from PTH-treated rats, and IGF-I was then considered as a potential mediator for the actions of PTH in bone tissue, possibly regulating the coupling of bone formation and bone resorption (Linkhart et al., 1989). However, PTH treatment of hypophysectomized (HYPOX) rats deficient in GH/IGF-I yielded conflicting results as to whether IGF-I is essential for the bone anabolic effects of PTH, possibly due to the poor physical condition of the rats and deficiencies in other bone-active hormones. Hock and Fonseca reported that the GH/IGF-I axis is essential for the bone anabolic actions of PTH (Hock, 1990). In contrast, Schmidt et al. performed a similar study in HYPOX rats and concluded that GH/IGF-I is not required for a PTH-induced stimulation of bone formation (Schmidt et al., 1995). Gunness and Hock reported that the PTH-induced increase in bone mass did not respond to supplementation with GH or IGF-I, when the rats were fed an energy-restricted or ad libitum diet (Gunness, 1995).
Dempster (1993) compared the effects of intermittent doses of PTH to the effects of mild primary hyperparathyroidism, in which there is an increase in the rate of bone remodeling, with newly formed BMUs (basic multicellular units) presenting more osteoblasts and osteoclasts, but with a balance in favor of bone formation. That could explain the dual mechanism of PTH, but there were still many questions to be answered concerning PTH mechanisms and the interaction of PTH with other drugs and/or other growth factors such as IGF-I, IGF-II, GH, and TGF β (Dempster et al., 1993). PTH activates osteoblasts, resulting in increased expression of genes with important roles in the production of growth factors, including IGF-I (Swarthout et al., 2002). Based on previous in vitro results, Miyakoshi et al. (2001) then worked with IGF-I knockout mice, finding that PTH treatment had no significant effects on serum bone formation markers, and did not increase bone mineral density, compared to their wild-type controls. His findings strongly suggested that IGF-I was required for the bone anabolic effects of PTH (Miyakoshi et al., 2001). In another study, two strains of mice, null for insulin receptor substrate-1 (IRS-1) and null for insulin receptor-2 (IRS-2), were treated with PTH and compared to their wild-type controls. The PTH anabolic effects were blunted in the IRS-1 -/- mice, indicating that the activation of IRS-1 was essential for PTH actions on bone (Yamaguchi et al., 2005).

In a comprehensive review, Jilka (2007) considered the increase in the number of matrix-synthesizing osteoblasts, increased osteoblastogenesis, attenuation of osteoblasts apoptosis, and activation of quiescent lining cells as explanations for the anabolic effects of PTH on bone. Nevertheless, the molecular and cellular mechanisms
underlying these skeletal effects are not completely clarified and the role IGF-I as a potential mediator for the bone anabolic effects of PTH is controversial (Jilka, 2007).

**Prostaglandin E₂**

Prostaglandins (PGs) are synthesized by bone cells and can affect both bone formation and bone resorption, acting as local and multifunctional regulators of skeletal metabolism. PGs are produced by cyclooxygenase, and their production is regulated by various local and systemic factors that ultimately induce cyclooxygenase -2 (COX-2) expression (Harada et al., 1995, Machwate et al., 2001). Through their anti-inflammatory actions, glucocorticoids can substantially inhibit PGs production, as they also inhibit COX-2 expression (Pilbeam et al., 2008).

Prostaglandins, especially prostaglandin E₂ (PGE₂), act on multiple receptors, and there are at least nine G-protein- coupled receptors (GPCRs) for PGs mediating their actions. The multiple receptors for PGE₂ are associated with four classes of GPCRs, named EP1, EP2, EP3 and EP4. Their ability to activate different signaling pathways makes it difficult to elucidate the effects on skeletal tissues (Pilbeam et al., 2008).

Prostaglandin E₂ (PGE₂) is the most abundant product in bone cells, and in vivo experiments showed that it increased bone mass in the metaphysis of the proximal and distal tibia and on endocortical and periosteal surfaces of the tibial shaft; it also stimulated bone formation and resorption evaluated by histomorphometric analyses (Lin et al., 1995). The anabolic effects of PGE₂ in cancellous bone could be observed in adult and even in aged rats (Ito et al., 1993, Cui et al., 2001).

PGE₂ is a potent inducer of cortical and trabecular bone formation in humans and animal models, with strong evidence that the bone anabolic effects are mediated by the EP4 receptor, as the administration of an antagonist to EP4 suppressed the action of
PGE$_2$ in increasing trabecular bone volume and bone formation indices. In addition, PGE$_2$ can improve fracture healing, stimulating both bone remodeling and angiogenesis, and induces the production of IGF-I, which modifies the rate of type I collagen synthesis (Pilbeam et al., 2008). Fracture healing and bone formation stimulated by PGE$_2$ are also correlated with mechanical loading. As observed by Jee and Ma (1997), the anabolic actions of PGE$_2$ are influenced by the impact of mechanical usage and the skeletal adaptation to this usage. Another influence was exerted by the source and abundance of osteoblast lineage cells (Jee and Ma, 1997).

PGE$_2$ stimulates the synthesis of IGF-I in osteoblast cultures from fetal rat bone, increasing IGF-I transcripts by 2.2 fold (McCarthy et al., 1991). In another study (Harada et al., 1995), in vivo and in vitro effects were observed; cancellous bone formation in the proximal tibial metaphysis was enhanced by PGE$_2$ treatment in ovariectomized rats, and in vitro, PGE$_2$ stimulated IGF-I expression. The IGF-I mRNA expression was correlated with osteogenesis (Harada et al., 1995). Despite the above evidence that PGE$_2$ upregulates gene expression for IGF-I in bone cells, the skeletal effects of PGE$_2$ in IGF-I deficient animal models have not been studied to date.

The GH/IGF-I Axis and Animal Models

Different animal models have been used to address the questions related to the GH/IGF-I axis and its influence on bone metabolism by inducing IGF-I deficiency. However, many of these surgically or genetically modified rodents have low survival rates, deficiencies in other bone-active hormones, and general health complications that might affect the interpretation of collected data.

**Hypophysectomized (HYPOX or HX) rats.** Hypophysectomy of immature rats results in decreased bone growth and osteopenia, due to decreased bone formation in
the presence of continued bone resorption. Growth hormone is considered the most effective growth stimulating pituitary hormone and has a dose-response effect on longitudinal bone growth in hypophysectomized rats (Thorngren and Hansson, 1975, Thorngren and Hansson, 1977, Thorngren et al., 1977, Schoenle et al., 1982).

However, hypophysectomized rats are markedly underweight and show diminished nonspecific immune responses with increased susceptibility to infections; as an example of this effect on the immune system, the macrophages from hypophysectomized rats exhibited 50% reduced capacity to kill Salmonella when compared to those derived from intact rats (Kooijman et al., 1996). These animals also present decreased erythropoiesis, resulting in anemia, leucopenia and thrombocytopenia (Yeh et al., 1999). GH and IGF-I administration can lead to resumption of growth and formation of red blood cells with increased incorporation of Fe (Kurtz et al., 1988). The intake of food is decreased in hypophysectomized rats, causing nutritional deficiencies as well. The muscle growth is also affected by the lack of GH; rats are less active in their cages, and this mechanical disuse leads to an overall effect similar to animals under partial immobilization or underloading (Yeh et al., 1997).

Pituitary hormones are important not only for longitudinal bone growth, but also for the radial growth of bone and maintaining cancellous bone balance (Isgaard et al., 1986, Kidder et al., 1997). The absence of hypophyseal hormones causes a marked decrease and delay in osteogenesis, affecting the quality of the newly formed bone, with decreased periosteal bone formation and depressed bone turnover, with more suppression in bone formation than in bone resorption, leading to an overall loss of cancellous bone mass (Yeh et al., 1999).
Nevertheless, hypophysectomized rats have been used mostly for studies of the effects of GH and IGF-I on longitudinal bone growth (Guler et al., 1988, Zapf et al., 1989, Turner et al., 2010). In addition, the effects of hypophysectomy are related to the age at the time of surgery and whether or not it is complete (Thorngren et al., 1980).

**Heterozygous (HET) mice.** Heterozygous mice have one functional IGF-I allele (IGF-I \(^+/-\)), and were developed by Powell-Braxton and collaborators in 1993 to study the IGF-I involvement in embryonic and postnatal growth. At that time, as IGF-I showed low levels in the embryo, it was thought to have an effective role only in postnatal growth and development. Compared to their wild-type littermates, the phenotype for the heterozygous animals included a 10 to 20% smaller size at birth and throughout their development, associated with a reduction in the size and weight of their organs, although there were no abnormalities in the analyzed tissues. There was no significant difference in the function of the growth plate, labeled with tetracycline, when compared to the wild-type mice. The IGF-I serum levels were approximately 37% lower, without affecting other serum components, and male and female heterozygotes were fertile and healthy, and could be intercrossed to generate homozygous IGF-I \(-/-\) mice (Powell-Braxton et al., 1993).

In another study with heterozygous mice, further analysis of bone morphometry revealed reduced femur length (4-6 %), reduced femur cross sectional area of cortical bone, marrow space and sub-periosteal area. Through microcomputed tomography, the femoral bone mineral density showed a decrease of 7-12% in both genders, when evaluated from 1-18 months, in comparison with wild-type mice. These findings were correlated to a 20-30% decrease in the IGF-I serum levels and body weight (10-20%).
The IGF-I protein levels were reduced by approximately 40% in the heterozygous cultures of neonatal calvarial osteoblasts, compared to the wild-type cells (He et al., 2006). Mohan and Baylink also observed that during puberty, the rate of gain in femoral bone mineral density was decreased by 25% in heterozygous IGF-I mice comparing with the control group, suggesting that IGF-I has an important role in bone acquisition during puberty (Mohan and Baylink, 2005).

The IFG-I haploinsufficiency was then considered as a suitable condition for studying the changes in bone that occur when IGF-I levels are only slightly decreased.

**IGF-I knockout mice.** Homozygous IGF-I knockout mice were also generated by the Powell-Braxton group, through the disruption of the mouse IGF-I gene and the intercross breeding of heterozygous IGF-I mice (IGF-I +/-). These animals were significantly smaller than their wild-type littermates, demonstrating a clear impairment of skeletal growth, with a 60% reduction in their body weight. They also showed a generalized muscular dystrophy, with decreases in muscle mass and maturation, mainly in the diaphragm, heart and tongue; atelectatic and less histologically organized lungs; hypoplastic epidermis, and no significant difference in bone development, between homozygous and wild-type mice. These changes could be correlated with the high degree of perinatal lethality observed (> 95%) that, once again, confirmed the important role of IGF-I in embryonic and postnatal growth. Initially, all IGF-I -/- mice were found dead at birth. However, approximately 5% of these mutant animals survived after birth up to four months, but were not fertile (Powell-Braxton et al., 1993).

When using histomorphometry, peripheral computerized tomography (pQCT) and microcomputerized tomography (μQCT) to evaluate more deeply the effects of this
mutation on bone tissue, it was found that 4 month old IGF-I knockout mice were 24 % of the body weight of the wild-type mice, and their tibiae were 28 % and the lumbar vertebrae (L1) 26 % of the size of the control animals' bones. In contrast, trabecular bone volume in the proximal tibia was increased in the IGF-I mice, compared to the wild-type, while the tibial bone periosteal formation rate and cortical thickness were decreased 27 % and 17 %, respectively. The growth plate and longitudinal bone growth were not evaluated in this study (Bikle et al., 2001).

In summary, the IGF-I knockout mouse has the advantage of complete absence of IGF-I, but is a difficult animal model to work with due to limited viability and the poor general health. In addition, IGF-I knockout mice have high serum levels of GH, which may complicate data interpretation.

**Liver IGF-I Deficient (LID) mice.** In 1999, Yakar and colleagues created another model to study the GH/IGF-I axis, by targeting the IGF-I gene specifically in the liver, obtaining liver IGF-I deficient (LID) mice (Yakar et al., 1999). These mutant animals have a complete ablation of liver *IGF-I* gene expression and were developed through the Cre/loxP System, a conditional knockout system of genetic recombination (Liu et al., 2000). This new mutation provided ways to test one of the statements of the Somatomedin Hypothesis, that implicated liver derived IGF-I as the main factor responsible for postnatal growth and skeletal development, acting as a mediator for GH. This new animal model suggested that local IGF-I could support postnatal growth and development. The reduction in IGF-I serum levels was about 75%, confirming the liver as the major source of circulating IGF-I. The remaining 25% of serum IGF-I represents the contribution of IGF-I synthesis by non-hepatic tissues. The GH serum levels,
however, showed markedly increased values in the LID mice, probably due to a negative feedback mechanism, in which the lack of circulating IGF-I leads to an increase in GH synthesis by the hypophysis. Surprisingly, the body length, as well as the femur length, was not significantly different between the LID mice and their controls. The LID mice did not show growth retardation, nor changes in skeletal IGF-I production, and were viable for at least 3-4 months. The average wet weights of targeted organs such as liver, heart, spleen and kidney, were similar between the LID and control mice. They were also fertile, and could give birth to normal sized pups (Yakar et al., 1999).

Liu also correlated the 75% reduction in serum IGF-I levels to a 50-100 fold decrease in IGF-I mRNA in the liver when compared to wild-type mice, while the body growth curves showed no difference with the wild-type mice, despite an increase in GH levels and a slight enlargement in the liver (Liu et al., 2000). On the other hand, when analyzing adult LID mice, there was a reduction in the periosteal circumference, femoral cross-sectional area, cortical thickness and total volumetric BMD, resulting in slender and more fragile bones with age (Yakar et al., 2009a).

Once established, the LID strain was used in cross-breeding with other mutant strains, e.g. knockout mice for the ALS (acid labile subunit), or mice with inactivation of IGF-IBP3 (IGF-I binding protein 3), producing double or triple mutant strains, according to the endocrine and/or skeletal phenotype to be investigated (Yakar et al., 2009b). In a recent review, more than 50 different mutant strains of mice were listed, according to their target action, genetic background, skeletal phenotype, and human counterpart (if there is a similar condition or disease in humans) (Yakar et al., 2010).
Dwarf rats. The dwarf rat (dw-4/dw-4) has potential as an animal model for studies of the effects of GH/IGF-I deficiency on bone structure and function. This mutation, inherited as an autosomal recessive, arose spontaneously in a breeding colony of Lewis rats at the Medical Research Council Cellular Immunology Unit, Sir William Dunn School of Pathology, Oxford, U.K., in 1985. Based on reports about their basic physiological characteristics by Charlton et al. (1988), the body growth in the mutant is retarded such that at 3 months of age, both males and females weigh approximately 40% less than their normal littermates, and continue to grow at a slower rate. GH synthesis in the pituitary is reduced to 10% of normal levels in males and 6% in females, and the GH mRNA levels are 20-25% of that normal, although the structural GH gene apparently is unaltered (Skottner et al., 1989, Charlton et al., 1988). Despite these hormonal changes, dwarf rats are outwardly healthy without any skeletal malformations, and therefore, appear to be a promising animal model for studies of GH/IGF-I deficiency.

Specific Aims

The proposed research focused on the role of circulating IGF-I in skeletal acquisition and whether or not low levels of serum IGF-I, sometimes found in osteopenic conditions, would affect the anabolic responses to PTH and PGE$_2$. The research project was, therefore, designed to evaluate, initially, the levels of serum IGF-I and the consequent changes in bone mass and indices of bone formation and resorption in GH/IGF-I deficient dwarf rats (dw-4/dw-4, autosomal recessive). Following this first analysis to establish the dwarf rat as an animal model for our studies, we tested the hypotheses that IGF-I acts as a potential mediator for the bone anabolic effects of PTH and PGE$_2$. In order to characterize the influence of this growth factor, the
responses to PTH and PGE\textsubscript{2} treatments were evaluated in two different strains of rats: dwarf rats and their background strain, Lewis rats. As mentioned, the dwarf mutation arose in an inbred colony of Lewis rats, thus allowing an appropriate direct comparison between the two strains (Charlton et al., 1988).

**Specific aim 1 – Evaluate the skeletal consequences of GH/IGF-I deficiency in dwarf rats by determining the IGF-I serum levels and its effects on bone structure and turnover.** We proposed the dwarf rat as an animal model to study the GH/IGF-I axis deficiency and its importance for normal bone metabolism. We measured the skeletal changes in both Lewis and dwarf rats and established baseline characteristics for further studies to compare the relative contribution of the GH/IGF-I axis to the bone anabolic effects of hormones and drugs. These changes were measured at the organ level by pQCT analyses, quantitative bone histomorphometry, and biomechanical testing.

**Specific Aim 2 – Evaluate the influence of GH/IGF-I deficiency on the effects of PTH treatment on bone mass and formation.** This experiment was designed to evaluate the influence of GH/IGF-I axis deficiency upon the bone anabolic effects of intermittent administration of PTH. We hypothesized that the ability of PTH to increase bone mass and stimulate bone formation would be diminished in dwarf rats with low IGF-I serum levels. This hypothesis was tested at the organ level by pQCT analyses, quantitative bone histomorphometry, and biomechanical testing.

**Specific Aim 3 – Evaluate the influence of GH/IGF-I deficiency on the effects of PGE\textsubscript{2} on bone mass and formation.** In this experiment the actions of PGE\textsubscript{2} on bone tissue were tested in dwarf rats with low IGF-I serum levels, with the objective of
clarifying whether or not this deficiency can impair the potential therapeutic use of PGE₂ for osteoporosis. We hypothesized that the ability of PGE₂ to increase bone mass and stimulate bone formation would be diminished in dwarf rats with low IGF-I serum levels. This hypothesis was tested at the organ level by pQCT analyses, quantitative bone histomorphometry, and biomechanical testing.

**Specific Aim 4 – Evaluate the influence of GH/IGF-I deficiency on the bone anabolic effects of PTH and PGE2 at the molecular level by analysis of gene expression.** In order to study the molecular mechanisms involved in the responses to PTH and PGE₂ treatments in IGF-I deficient dwarf rats, we evaluated, by real time RT-PCR, the expression of genes involved in bone formation and bone resorption, as listed below:

- Bone formation: IGF-I, Collagen type I, Osteocalcin, Osterix, and Sclerostin.
- Bone resorption: RANKL and Osteoprotegerin (OPG).

In addition, the gene expression of IGF-I was also evaluated in hepatic tissue samples, to differentiate the expression of circulating IGF-I produced by the liver, from the local IGF-I synthesized by bone cells.
CHAPTER 2
THE EFFECTS OF A DEPRESSED GH/IGF-I AXIS ON BONE STRUCTURE AND BONE TURNOVER IN DWARF RATS

Introduction

The laboratory rat *Ratus norvegicus* has been a primary model in biomedical research, and a constantly increasing number of mutant strains have been providing disease-targeted models for scientific investigations (Steen et al., 1999, Pearce et al., 2007, Lelovas et al., 2008). Particularly in bone research, the rat has been used to successfully reproduce osteoporosis/osteopenic conditions and growth related diseases, and even though it does not exhibit the same pattern of Haversian systems in cortical bone as seen in humans and higher mammals, it still provides data that can be translated to other species (Lelovas et al., 2008). Moreover, the amount of available information about the rat skeleton and the rat genome, protocols for inducing osteopenia, orthopedic testing techniques, and reliable information that can be applied to different pathological conditions, make the rat an adequate animal model in this field of science, having contributed to many therapeutic advances in bone disease management.

GH stimulates the synthesis of IGF-I in the liver, as well as in almost every tissue, including bone, and most of its anabolic action is mediated by IGF-I, an important growth factor in the skeleton. IGF-I has multiple physiological functions, acting through endocrine, paracrine and autocrine pathways, and is essential for both embryonic and postnatal growth (Powell-Braxton et al., 1993, Liu et al., 1993, Bikle et al., 2001, Iida et al., 2005). The use of animal models has been crucial for understanding the numerous mechanisms and molecular pathways involved in the GH/IGF-I axis.
The dwarf rat has been used as an animal model in other studies to evaluate growth responses to GH and IGF-I (Skottner et al., 1989), and as previously mentioned, this model could allow a direct comparison of bone parameters with its background strain, the Lewis rat. Although GH synthesis is substantially reduced in dwarf rats, differently from heterozygous and/or knockout animals, these rats are healthy without skeletal malformations, and the concentrations of other trophic hormones such as luteinizing hormone (LH), thyroid-stimulating hormone (TSH), prolactin (PRL) and adrenocorticotrophic hormone (ACTH), do not differ from unaffected heterozygotes (Charlton et al., 1988). In addition, bone mass, bone growth, and osteoblast activity are decreased in dwarf rats. These characteristics make dwarf rats suitable for the study of osteoporosis, as well as other osteopenic syndromes in which the GH/IGF-I serum levels are decreased but not abolished, as seen in most clinical conditions. The use of this mutant would mimic the GH/IGF-I deficiency as it appears in clinical cases of postmenopausal osteopenia and other bone loss conditions, such as anorexia nervosa, corticoid therapy induced osteopenia, and chemotherapy.

Lange et al. (2004) also found that there was a qualitative and quantitative difference, between dwarf and Lewis rats, in the morphology and arrangement of collagen microfibrils of femurs evaluated by SEM (scanning electron microscopy) (Lange et al., 2004). IGF-I and IGFBP3 were significantly decreased in dwarf rats, compared to the control group, in addition to decreased bone biomechanical properties: ultimate stress, stiffness and energy at load. These findings were correlated with the decreased strength and increased occurrence of fractures observed in GH deficient patients.
For all the characteristics presented in Chapter 1, we consider that the dwarf rat is suitable to address the importance of IGF-I for the effectiveness of certain pharmacological agents in stimulating bone formation. The present study was, therefore, designed to evaluate, initially, the levels of serum IGF-I and the consequent changes in bone mass and levels of bone formation and resorption in GH/IGF-I deficient dwarf rats (dw-4/dw-4, autosomal recessive). For this study, two hypotheses were tested:

1. The GH/IGF-I deficiency in dwarf rats results in a significant decrease in IGF-I serum levels, compared to those observed in HYPOX rats (85%), LID mice (75%) and HET mice (20-30%).
2. The GH/IGF-I deficiency would similarly reduce body weight, decrease bone growth and cause osteopenia as observed in the animal models mentioned above.

Materials and Methods

Animal Model

In order to compare the two different strains of rats: dwarf rats and their background strain, we used gender and age-matched Lewis controls. The animals, five week old female dwarf and Lewis rats, were obtained from the same supplier, Harlan Laboratories (UK). They were housed two per cage, with relative humidity, air quality, illumination (12h light/12h dark cycles), and temperature controlled according to the criteria established by the Institute for Laboratory Animal Research and the National Research Council (2011). The protocol for use of rats in this research project was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Florida.
Experimental Design

Both groups of Lewis and dwarf rats consisted of 13 animals. Since dwarfism is not reliably noticed until 4-5 weeks of age, the rats were maintained in the same nutritional and environmental conditions for four weeks before starting treatment. During this period, all rats had their clinical status evaluated daily and were weighed weekly.

Vehicle Treatment

Starting at nine weeks of age, the rats were injected daily, subcutaneously, with vehicle solution, for two weeks. The preparation of the vehicle solution involved:

- Dissolving 0.1 ml of concentrated hydrochloric acid in 1000 ml pyrogen free distilled water, to get a 0.001N HCl solution.
- Addition of 97.9 ml of sterile saline (0.9g NaCl) to 0.1 ml of the previous solution, resulting in a 0.015M NaCl solution, followed by filtration with #22 Milipore filter.
- Addition of 2 mL of heat inactivated rat serum.

Bone Formation Markers

All rats received intraperitoneal injections of fluorochrome compounds, declomycin and calcein (Sigma Chemical Co., St. Louis, MO), at a dose of 15 mg/kg body weight, ten and three days prior to euthanasia, respectively, in order to label actively forming bone surfaces.

Euthanasia and Tissue Sample Distribution

The animals were euthanized in accordance with the AVMA (American Veterinary Medical Association) Guidelines on Euthanasia- 2007, and following the protocol already approved by the IACUC. They were anesthetized with ketamine (Ketaset® – Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (AnaSed® – Lloyd Laboratories, Shenandoah, IA), at doses of 50 and 10 mg/kg, respectively. Once a deep level of
anesthesia was achieved, exanguination from the abdominal aorta was performed, followed by cervical dislocation to confirm death. Serum samples were stored at -80°C for future analyses. The animal carcasses were examined for macroscopic pathological changes.

Both tibiae, right femur and left distal femur, the first, second, third and fourth lumbar vertebrae, and the fifth and sixth caudal vertebrae, as well as the liver, were collected at the time of necropsy. All bone specimens were stripped of musculature. The tibiae, first and second lumbar vertebrae, and the caudal vertebrae were placed in 10% phosphate-buffered formalin (pH 7.4) for 24 hours. These bone samples were then transferred to 70% ethanol, and further processed undecalcified for quantitative bone histomorphometry.

The right femora were placed in 70% ethanol until evaluated for total length and bone mineral by peripheral quantitative computerized tomography.

The left distal femora and liver samples were snap frozen in liquid nitrogen, and transferred to a -80°C freezer for future gene expression analyses. The third and fourth lumbar vertebrae were also stored in a -80°C freezer for biomechanical analysis.

**IGF-I Serum Levels**

The ELISA (Enzyme-Linked Immuno Sorbent Assay) method was performed to obtain the IGF-I serum levels for all animals. IGF-I, although highly conserved between species, shows some differences when comparing human to rat or mouse IGF-I. There are three amino acid substitutions that distinguish rat from human IGF-I, and although there are only a small number of substitutions, most human IGF-I assays are not able to measure rat or mouse IGF-I. The antibody must recognize the specific peptide of the species being analyzed, and for these reasons, we used the Rat/Mouse IGF-I ELISA kit.
(IDS - Immuno Diagnostic Systems), a specific immunoenzymometric assay (IEMA) for quantitative determination of Insulin-like Growth Factor I in rat and mouse serum or plasma.

The serum samples were incubated briefly with a reagent to inactivate binding proteins, and then diluted for the assay. A purified monoclonal anti-Rat IGF-I is coated onto the inner surface of microtitre wells, where the samples were then incubated together with biotinylated polyclonal rabbit anti-Rat IGF-I, and shaken for two hours at room temperature. The wells were then washed and enzyme (horse radish peroxidase) labelled avidin was added, binding to the biotin complex. After a further wash, a simple component chromogenic substrate (a formulation of tetramethyl-benzidine) was added to develop color. The absorbance (at 450 nm, with reference 620 nm) of the reaction, for each well, was read in a microtitre plate reader, with color intensity being proportional to the amount of rat IGF-I present in the samples. The analysis was achieved with the Ascent Multiscan ELISA Software. The mean absorbance for each calibrator was used to generate a calibration curve, and obtain the slope of the equation to calculate the concentration of serum IGF-I in ng/mL.

**Evaluation of Body Weight and Femoral Length**

The evaluation of body weight and femoral length were performed as an initial approach to verify the effects of GH/IGF-I deficiency on the overall development and growth of dwarf rats, in comparison to Lewis rats. For this purpose, the animals were weighed, since their arrival in the animal care facility, with the same precision scale. After necropsy, the length of the right femur was measured with an electronic digital caliper (Fisher Scientific), considering the femoral head and the femoral condyles as proximal and distal reference points for measurement, respectively.
Peripheral Quantitative Computerized Tomography

Peripheral Quantitative Computerized Tomography (pQCT) is a non-invasive, sensitive and reproducible method to monitor changes in cancellous and cortical bone mass, bone density, and geometric properties. Moreover, this technology determines some indicators of bone properties that are relevant to bone strength: mass, mechanical quality and spatial distribution of bone material (Ferretti, 1999). Its precision and accuracy can be compared to histology and microcomputed tomography (µCT) (Schmidt et al., 2003), and yet, as it measures volumetric density, it should not be affected by bone size, as with DXA (Dual X-energy x-ray absorptiometry), a method that measures areal bone mineral density. The pQCT captures a 3-dimensional image, and allows the analysis of cortical and cancellous bone separately. This method has an accuracy of 92 to 98% and should be viewed as a complimentary technique to static and dynamic histomorphometry (Lelovas et al., 2008).

The right femurs were scanned and analyzed by pQCT with a Stratec XCT Research M instrument (Norland Medical Systems, Fort Atkinson, WI). The difference in femur length between the two strains was considered when determining the region of interest at both sites, the distal femoral metaphysis, which is rich in trabecular bone, and the femoral shaft, which is composed entirely of cortical bone. Trabecular bone measurements were taken at a distance of 5mm and 4mm from the distal end of the femur, for Lewis and dwarf rats, respectively.

Similarly, when evaluating cortical bone in the femoral shaft, the images were obtained at distances of 16mm and 14mm from the distal end of the femur in Lewis and dwarf rats, respectively.
Bone structural parameters measured by pQCT at the distal femoral metaphysis were:

1. Total bone mineral content (total BMC, mg/mm);
2. Total bone mineral density (total BMD, mg/cm$^3$);
3. Trabecular bone mineral content (trabecular BMC, mg/mm);
4. Trabecular bone mineral density (trabecular BMD, mg/cm$^3$);
5. Total area (mm$^2$);
6. Trabecular area (mm$^2$).

Bone structural parameters measured at the femoral diaphysis (shaft) were:

1. Total bone mineral content (total BMC, mg/mm);
2. Total bone mineral density (total BMD, mg/cm$^3$);
3. Cortical bone mineral content (cortical BMC, mg/mm);
4. Cortical bone mineral density (cortical BMD, mg/cm$^3$);
5. Cortical area (mm$^2$);
6. Cortical thickness (mm);
7. Periosteal circumference (mm);
8. Endosteal (endocortical) circumference (mm).

**Bone Histomorphometry**

Bone histomorphometry is a histological method that provides quantitative data about bone structure/organization and bone remodeling, by using a computer-assisted analysis of microscopic images. It accurately quantifies the level of cellular activity and bone mass from undecalcified bone biopsy specimens or bone samples, representing an important tool in both clinical assessment of bone health status and research. Fluorochrome markers, such as tetracycline, declomycin, and calcein, are also used to obtain dynamic or kinetic parameters that yield important information on bone formation.

The right proximal tibia and first lumbar vertebra were collected from each rat, dissected free of surrounding soft tissue, dehydrated in increasing concentrations of ethanol (70 %, 95%, and 100%), and processed undecalcified in modified methyl metacrylate (Baron et al., 1983). The samples were sectioned longitudinally with Jung
2065 and 2165 microtomes (Leica Corp., Rockleigh, NJ) at thicknesses of 4 and 8 μm. The thinner sections were stained according to the von Kossa method with a tetrachrome counterstain (Polysciences, Warrington, PA), whereas the 8 μm-thick sections remained unstained for collection of fluorochrome-based data.

Bone measurements were performed in cancellous bone tissue of the proximal tibial metaphysis beginning at a distance of 1 mm from the growth plate-metaphyseal junction to exclude the primary spongiosa. The region of interest within the first lumbar vertebral body excluded the primary spongiosa within 0.5 mm of the cranial and caudal growth plates. The bone variables were measured with the Osteomeasure System (Osteometrics, Atlanta, GA) and the Bioquant Bone Morphometry System (R&M Biometrics Corp., Nashville, TN), bone-specific computer image analysis systems, and using a Nikon microscope equipped with ultraviolet lighting. The bone histomorphometric indices were described according to the standardized nomenclature, symbols and units proposed by Parfitt et al. (1987) for the ASBMR (American Society of Bone and Mineral Research) Histomorphometry Nomenclature Committee (Parfit et al., 1987), and included:

1. Cancellous Bone Volume (BV/TV, %): percent of cancellous bone in the tissue area analyzed.
2. Trabecular Width (Tb.Wi, μm): mean width of trabeculae.
3. Trabecular Number (Tb.N, #/mm): number of trabeculae per unit distance.
5. Osteoid Surface (OS/BS, %): percent of bone surface covered by osteoid layer.
6. Osteoblast Surface (Ob.S/BS, %): percent of bone surface with adjacent osteoblasts.
7. Osteoclast Surface (Oc.S/BS, %): percent of bone surface with adjacent osteoclasts.

In addition, the following fluorochrome-based data were collected from unstained, 8 µm-thick bone sections with the same quantitative systems described above, to derive the kinetic or dynamic variables of bone formation:

1. Rate of Longitudinal Bone Growth (µm/day): rate at which the proximal tibia grows in length each day.

2. Mineralizing Surface (MS/BS, %): percent of bone surface with fluorochrome labels (double-labeled plus half the single-labeled), representing active mineralization and bone formation.

3. Mineral Apposition Rate (MAR, µm/day): distance between the labels divided by the time interval between their administration. This is an index of osteoblastic activity.

4. Bone Formation Rate (BFR/BS, $10^{-2} \mu m^3/\mu m^2/day$): amount of bone formed in unit time per unit of bone surface. It is calculated by multiplication of mineral apposition rate and mineralizing surface.

For the cortical analyses, the right tibia from each animal was dissected free of muscle, cut in half cross-sectionally with a hand-held saw, and placed in 10% phosphate-buffered formalin for 24 hours for tissue fixation, followed by storage in 70% ethanol. The distal halves of the tibiae were dehydrated and defatted in 10 changes of 100% ethanol and 10 changes of acetone (at least 2 hours per change), followed by embedding undecalcified in a styrene monomer that polymerizes into a polyester resin (Tap Plastics, San Jose, CA). The tibial diaphyses were sawed 1-2 mm proximal to the tibio-fibular junction into approximately 100 µm-thick cross sections with an Isomet low-speed saw (Buehler, Lake Bluff, IL). These cross sections of cortical bone were then ground to a thickness of 50 µm between roughened glass plates prior to the histomorphometric measurements also performed with the Osteomeasure System.
(Osteometrics, Atlanta, GA). The following structural measurements were performed in one cross section per animal at a magnification of 2X.

1. Cortical Bone Tissue Area (mm$^2$): cortical bone area plus the bone marrow area.
2. Marrow Area (mm$^2$): area of the bone marrow cavity.
3. Cortical Bone Area (mm$^2$): calculated by subtracting the marrow area from cortical bone tissue area.
4. Periosteal Perimeter (mm): distance around the outer surface of cortical bone.
5. Endocortical Perimeter (mm): distance around the inner surface of cortical bone.
6. Cortical Width (μm): measured by averaging the distance from the periosteal surface to the endocortical surface at 8 different locations around the bone.

Kinetic values for periosteal and endocortical bone formation were also obtained through collection of double and single label fluorochrome-based data, measured at 200X under UV illumination, as follows:

1. Periosteal Mineralizing Surface (%): calculated as the percent of double-labeled surface plus one half the percent of single labeled surface.
2. Periosteal Mineral Apposition Rate (μm/day): calculated by dividing the interlabel distance by the time interval between administration of labels (7 days).
3. Periosteal Bone Formation Rate ($10^{-2} \mu m^3/\mu m^2/day$): calculated by multiplying the mineralizing surface by the mineral apposition rate.
4. Endocortical Mineralizing Surface (%);
5. Endocortical Mineral Apposition Rate (μm/day);
6. Endocortical Bone Formation Rate ($10^{-2} \mu m^3/\mu m^2/day$).

Biomechanical Testing

An increase in bone mass does not always translate into a decrease in the occurrence of fractures, making it important to also evaluate the mechanical strength of bones. However, the most common tests, such as three-point bending, 4-point bending and torsion, assess the strength of the diaphysis of long bones, at sites where
osteoporotic fractures occur less frequently. For this reason, we performed compression testing of the lumbar vertebral body, a more common site for osteoporotic fractures, with an 858 MTS Mini Bionix II machine.

At necropsy, lumbar vertebrae 3 and 4 were snap frozen, and stored at -80°C until ready to be tested, when they were thawed at room temperature. In order to ensure uniformity between specimens, and to prevent lateral deformation, each vertebral body was cut with parallel edges, making sure there was no remaining tissue from intervertebral disks. This was accomplished using a custom machined wood block to hold the specimens and an 80/20 (Columbia City, IN) adjustable swing-arm with an attached Dremel Multitool (Mount Prospect, IL) for cutting the parallel surfaces. Diamond cutting blades were used for the most precise cut. The vertebrae were irrigated with cooling saline during the cutting process to prevent a change in the molecular structure of the tissue due to heat.

After cutting, the height of each vertebral body was measured with an electronic digital caliper (Fisher Scientific), and photographed with a high-resolution digital camera (Nikon Inc., Melville, NY). Afterwards, the images were analyzed with image software (Image J) to obtain the area of the vertebral body. Compression testing was done with the 858 MTS Mini Bionix II machine (Eden Prairie, MN). Each specimen was placed onto a flat steel plate and then loaded in compression until failure at a rate of 3 mm/sec. All failure data were recorded internally by the MTS load cell and then exported to a spreadsheet. Failure was defined by a decrease in detected force on the load cell, or a yield point. Maximum stress was calculated using the area measurements from the images captured earlier and the maximum force recorded by the MTS. Stiffness was
calculated using the height measurements and the maximum force recorded by the MTS. In summary, the parameters used for biomechanical analysis of the vertebrae were:

1. Load (N)
2. Stress (N*mm² or MPa)
3. Stiffness (N/mm)

**Statistical Analyses**

Data are presented as the mean ± SD for each group, unless stated otherwise. Statistical differences among groups were determined by analysis of variance (ANOVA), followed by a multiple comparison test (Scheffe post hoc analysis, StatView - SAS Software Institute Inc.). Differences were considered significant at P<0.05. Alternatively, if the data were not normally distributed, statistical differences were evaluated with the non-parametric Kruskal Wallis test, which is valid for both normal and non-normal data distributions.

**Results**

**Body Weight and Femoral Length**

Visible phenotypic differences between Lewis and dwarf rats could be observed before the beginning of treatment, with Lewis rats showing more increase in body size and weight (Figures 2-1 and 2-2), as expected. At the time of euthanasia, the mean body weight of the Lewis rats was 52.9 % greater than the dwarf rats (187.6 ± 10.9 g vs. 122.7 ± 5.6 g, P<0.0001) as seen in Figure 2-3, and their right femurs were 14.8 % longer (32.32 ± 0.42 mm vs. 27.28 ± 0.32 mm).

**IGF-I Serum Levels**

Serum IGF-I concentration values, calculated in ng/mL, based on the equation of the calibration curve: y = 0.140 + (0.000230 * X), as seen in Figure 2-4, revealed that the
serum levels of IGF-I were nearly 2.5-fold lower in dwarf rats compared with Lewis rats (353.36 ± 153.15 ng/mL vs. 845.22 ± 169.45 ng/mL), as seen in Figure 2-5.

**Peripheral Quantitative Computerized Tomography**

The pQCT analyses of the distal femoral metaphysis revealed that bone structural values for the parameters total BMC, total BMD, trabecular BMC, and trabecular BMD were significantly lower in dwarf rats compared to Lewis rats (P<0.0001), as shown in Figures 2-6 to 2-11.

All the values obtained at the femoral shafts for total BMC, total BMD, cortical BMC, cortical area, cortical thickness, periosteal and endocortical circumferences were also significantly lower in dwarf rats, with the exception of cortical BMD (Figures 2-12 to 2-19).

**Histomorphometric Findings**

**Cancellous bone measurements in lumbar vertebrae**

Cancellous bone volume was significantly lower in the dwarf rats than in the Lewis rats (15.99 ±4.40 % vs. 26.40 ± 6.44 %, P<0.0001), and this cancellous osteopenia was associated with decreased trabecular number (5.44 ± 0.86 #/mm vs. 6.57 ± 1.01 #/mm, P<0.0053) and width (34.86 ± 5.18 µm vs. 48.50 ± 12.30 µm, P<0.0012), and increased trabecular separation (158.70 ± 29.6 µm vs. 115.18 ± 26.28 µm, P< 0.0006), as observed in Figures 2-20 to 2-23.

When considering cancellous bone cellular surface parameters (Figures 2-24 to 2-26), there were no statistically significant differences between the two groups; in fact, the mean values for osteoclast surface, an index of bone resorption, were nearly identical in Lewis and dwarf rats.
Although mean values for osteoblast and osteoid surfaces were not significantly different between the two groups, when analyzing bone kinetic variables (Figures 2-27 to 2-29), it was found that the cancellous bone mineral apposition rate, an index of osteoblast activity, was significantly lower in dwarf rats compared to Lewis rats (0.88 ± 0.09 µm/d vs. 1.07 ± 0.12 µm/d, P<0.0001). In addition, the mineralizing surface in the dwarf rats (14.03 ± 6.78%) was also significantly lower than that observed in the Lewis rats (29.96 ± 6.05%, P<0.0001). For bone formation rate, the mean value for the dwarf rats (12.15 ± 5.87 10⁻² µm³/µm²/d) was lower (P<0.0001) than that of Lewis rats (32.37 ± 8.34 10⁻² µm³/µm²/d).

**Cancellous bone measurements in proximal tibiae**

As observed before, dwarf rats showed lower values for body weight and femoral length, and not surprisingly, tibial longitudinal bone growth was three-fold lower in the dwarf rats compared to the Lewis rats (27.29 ± 8.67 µm/d vs. 70.88 ± 5.55 µm/d, P<0.0001) as seen in Figure 2-30. The effect of a depressed GH/IGF-I axis on cancellous bone structure is even more noticeable in the histologic images of proximal tibial sections from dwarf and Lewis rats in Figure 2-31. Among the cancellous bone structural values for the tibiae (Figures 2-32 to 2-35), cancellous bone volume (2.77 ± 1.83 % vs. 19.4 ± 2.75 %, P<0.0001), trabecular number (1.52 ± 0.93 #/mm vs. 6.19 ± 0.90 #/mm, P<0.0001), and trabecular width (21.35 ± 3.0 µm vs. 37.99 ± 6.3 µm, P<0.0001), were significantly lower in the dwarf rats compared to the Lewis rats, respectively. As a consequence, the trabecular separation values were higher in the dwarf rats than in the control strain (1738.88 ± 2241.08 µm vs. 133.26 ± 23.88 µm, P<0.016).
Cortical bone measurements in the tibial diaphysis

Dwarf mutation's effects could also be observed in the left tibial diaphyses, as shown in Figures 2-36 to 2-41. Dwarf rats' cortical bone structural measurements were significantly lower than those of Lewis rats, including the total bone tissue area (2.49 ± 0.16 mm² vs. 3.67 ± 0.27 mm², P<0.0001), cortical area (1.95 ± 0.10 mm² vs. 3.04 ± 0.20 mm², P<0.0001), marrow area (0.54 ± 0.08 mm² vs. 0.63 ± 0.10 mm², P<0.02), periosteal perimeter (5.86 ± 0.17 mm vs. 7.06 ± 0.30 mm, P<0.0001), endocortical perimeter (2.69 ± 0.20 mm vs. 2.89 ± 0.24 mm, P<0.044), and cortical width (504.55 ± 27.61 μm vs. 669.75 ± 30.92 μm, P<0.0001).

Regarding cortical bone kinetic indices, the periosteal mineralizing surface (49.44 ± 17.79 % vs. 84.02 ± 25.25 %, P<0.0007), periosteal mineral apposition rate (1.15 ± 0.30 μm/d vs. 2.40 ± 0.52 μm/d, P<0.0001), and periosteal bone formation rate (60.38 ± 31.68 10⁻² μm³/μm²/d vs. 211.41 ± 73.48, P<0.0001), as well as the endocortical mineralizing surface (50.52 ± 19.77 % vs. 82.29 ± 21.95 %, P<0.0015), endocortical mineral apposition rate (0.83 ± 0.18 μm/d vs. 1.49 ± 0.55 μm/d, P<0.0008), and endocortical bone formation rate (42.82 ± 19.56 10⁻² μm³/μm²/d vs. 128.94 ± 59.64 10⁻² μm³/μm²/d, P<0.0001), were all significantly lower in dwarf rats than in Lewis rats, respectively, as shown in Figures 2-42 to 2-47.

Biomechanical Testing

The areas of the lumbar vertebrae were measured in the obtained photographs with Image J software, as shown in Figures 2-48 and 2-49. These values (not shown) were used only for calculation of the stress parameter, and the heights of the vertebrae (data not shown) were used only to calculate stiffness. Biomechanical load values were significantly lower in dwarf rats compared to Lewis rats (P=0.038), but no differences
were observed for biomechanical stress and stiffness between the two groups, as seen in Figures 2-49 to 2-51.

**Discussion**

**IGF-I Serum Levels**

The hypophysectomized rat was considered, at first, the classical model to study GH actions and deficiencies, exhibiting IGF-I serum levels 85% lower than intact animals. The dwarf rats presented a 60% reduction in the IGF-I serum levels when compared to Lewis rats. Obviously, the effect was not as dramatic as that observed in the hypophysectomized rats. However, in addition to GH, hypophysectomized rats lack all other hormones synthesized by the hypophysis, that also act on growth and bone metabolism. Due to this abrogation of other bone acting hormones and related growth factors, it is difficult to interpret data obtained from hypophysectomized rats. Another drawback to be considered is the possibility of remaining hypophyseal tissue in the sella turcica. Thorngren et al. screened the completeness of hypophysectomy through serial microscopic sections of the sella turcica and measurements of longitudinal bone growth by tetracycline labeling, 15 days after surgery (Thorngren et al., 1980). The longitudinal bone growth parameters were correlated with hypophyseal tissue remnants in the sella turcica (Thorngren et al., 1980). In contrast, the recessive mutation of the dwarf rats results in partial deficiency in GH production, but with normal levels of the other trophic hormones such as luteinizing hormone (LH), thyroid-stimulating hormone (TSH), prolactin (PRL) and adrenocorticotropic hormone (ACTH) (Skottner et al., 1989). Therefore, the dwarf rat has fewer variables in systemic hormone circulation to potentially complicate data interpretation.
The circulating IGF-I values obtained for the dwarf rats were also higher than those expressed by LID mice (75% reduction) and by IGF-I knockout mice, whose levels are at or even below detection levels (22 ng/mL). However, as mentioned before, only 5% of the IGF-I knockout mice survive up to four months (Powell-Braxton et al., 1993). LID mice, and other transgenic strains developed to elucidate the contributions of local and systemic IGF-I, are obviously a great tool in terms of elucidating the molecular pathways involved in the GH/IGF-I axis, but the markedly higher serum GH levels in the LID mice may complicate data interpretation.

The heterozygous (HET) mice have their IGF-I serum levels reduced by only 20-30% when compared to their wild-type littermates (He et al., 2006), a reduction that may be considered too small to compare to the 54% decreased IGF-I serum levels found in patients with anorexia nervosa or the 73% drop in IGF-I serum levels in children with type I diabetes mellitus (Gianotti et al., 1998, Grinspoon et al., 1999).

**Body Size and Development**

When analyzing the development and body size of Lewis and dwarf rats through their weight and femoral length, we verified that the body weight of Lewis rats was 52.9% greater than that of the dwarf rats, and their left femurs were 14.8% longer compared to the dwarf rats. These results were comparable to other rodent models of IGF-I deficiency, but were not evident at birth; in fact, the observed changes were noticed after 4-5 weeks of age, demonstrating that postnatal skeletal development and bone growth were clearly affected by the dwarf mutation. In contrast, IGF-I knockout mice were born at 60% of normal weight, and postnatal growth curves indicated that the surviving IGF-I (-/-) mutants continued to grow with a retarded rate after birth in comparison to their controls, and were 30% of normal weight as adults (Baker et al.,
When knockout mice were first described in 1993 by Baker, Liu, and Powell-Braxton, 80 to 95% lethality was reported. Liu (1993) reported that 10 to 68% of IGF-I (-/-) mutants survived, and attributed the difference in survival rates to the genetic background, whereas Powell-Braxton (1993) considered the position where the \(IGF-I\) gene was interrupted as the possible source of difference (Liu et al., 1993, Powell-Braxton et al., 1993). Bikle (2001) compared the skeletal structure of IGF-I deficient, heterozygous and homozygous, mice to their wild-type controls, and by 12 weeks of age, female and male IGF-I knockout mice were approximately 30 and 20%, respectively, of the weight of their wild-type controls (Bikle et al., 2001). Recently, in a comprehensive study about IGF-I mutant mouse models, Yakar (2010) considered total inactivation of the \(IGF-I\) gene to result in 80% perinatal lethality, with surviving pups 50% smaller than the wild-type controls (Yakar et al., 2010).

**Peripheral Quantitative Computerized Tomography**

Osteopenia in human patients routinely is evaluated by measurements of bone mineral density by the DXA method, and is confirmed when values are under 2.5 standard deviations from normal. In this research project, the pQCT parameters for Lewis rats were considered normal for evaluation purposes. There was a clear osteopenic condition in dwarf rats revealed by the pQCT data. The total and trabecular cancellous bone mineral density were significantly decreased in the distal femoral metaphysis, as well as reduced values for bone mineral content (\(p<0.0001\)). The low BMD values in femoral cancellous bone in dwarf rats (12%) could be compared to those observed in young heterozygous mice (7-12%) (Mohan and Baylink, 2005, He et al., 2006).
The only parameter that was not significantly lower in the dwarf rats was cortical bone mineral density. A possible explanation for this observation comes from the fact that the animals in our study were young, and bone modeling related to growth and changes in bone shape due to mechanical loading were still occurring. Since Wolff's law first introduced the concept that “every change in the form and function of a bone, or its function alone, is followed by certain definite changes in its external conformation” (Stedman, 1990), several studies examined the various aspects of bone responses to loading in anatomical studies of paleontological skeletal remains (Pearson and Lieberman, 2004, Ruff, 2005), in vitro (Klein-Nulend et al., 1995, Mullender et al., 2004), and in vivo, mainly in clinical studies. Most of these clinical studies support the general conclusion that cortical bone responses to loading occur primarily in the juvenile state, leading to macroscopic effects on long bone cross-sections and increased bone density, and that there are age-related differences in the ability of bone to respond to exercise and load (Prior et al., 1996, Ruff, 2003, Pearson and Lieberman, 2004, Greene and Naughton, 2006, Judex et al., 2008). The majority of cells in cortical bone are osteocytes. Although the molecular mechanisms and pathways involved in mechanosensation and mechanotransduction are complex and not yet identified, osteocytes are considered mechanosensors of bone (Klein-Nulend et al., 1995, Judex et al., 2008, Klein-Nulend and Bonewald, 2008). In addition, cortical remodeling would not yet be taking place during this experiment, as the transition time from modeling to remodeling in the rat skeleton would be around 3 to 6 months for endocortical bone, in the lumbar vertebra, and 9 to 12 months in the proximal tibial metaphysis (Lelovas et al., 2008).
Bone Histomorphometry and Biomechanics

The histomorphometric findings were consistent with the pQCT data. The lower values in dwarf rats for total BMD and trabecular BMD in the distal femoral metaphysis were fully consistent with the low cancellous bone volume, decreased trabecular number and width (p<0.0001), and increased trabecular separation (p<0.0001) observed in the structural analysis of histologic sections of the proximal tibial metaphysis and lumbar vertebral body. The lower vertebral bone mass resulted in decreased bone strength in dwarf rats, as indicated by significantly lower load to failure in the lumbar vertebral body of these IGF-I deficient animals. Although bone cellular surface parameters (osteoblast and osteoclast surfaces) did not show significant differences between Lewis and dwarf rats, dynamic indices of bone formation at the cancellous, periosteal, and endocortical bone surfaces were all significantly decreased in dwarf rats compared to Lewis rats. These findings suggest that the osteopenia detected in dwarf rats is due primarily to an inhibition of bone formation. Since osteoclast surface was nearly identical in dwarf and Lewis rats, bone resorption did not appear to be affected by IGF-I deficiency. Yet, the dwarf rat expressed an evident osteopenic phenotype, characterizing it as a good model to reproduce the observed osteopenia in IGF-I deficiency related conditions.

Conclusions

The results addressed Specific Aim 1, and the major findings were:

1. The dwarf rat expressed significantly decreased IGF-I serum levels compared to its background strain, representing a major reduction of 60 % in circulating IGF-I. Therefore, the dwarf rat is a reliable model to study the physiological changes seen in IGF-I deficiency.

2. GH/IGF-I deficiency in the dwarf rat has profound negative effects on bone growth, accumulation of bone mass, bone strength, and bone formation in both
cortical and cancellous bone. For this reason, it is a valid animal model to study the osteopenic effects caused by this deficiency on bone structure and metabolism.

3. Since IGF-I may mediate the skeletal effects of bone anabolic agents, the dwarf rat is also a promising animal model for studies of these interactions.
Figure 2-1. The two strains of Lewis (right) and dwarf (left) rats during their clinical evaluation. Photo courtesy of Dr. Ana Cristina F. Bassit

Figure 2-2. Dwarf rat (right), showing inferior size and overall development when compared to Lewis rat (left). Photo courtesy of Dr. Ana Cristina F. Bassit
Figure 2-3. Body weight (g). There was a statistically significant difference between Lewis and dwarf rats (P<0.0001)
Figure 2-4. IGF-I Serum Levels. The calibration curve and slope equation for obtaining the concentration levels for IGF-I in ng/mL

\[ y = 0.0002x + 0.1401 \]
\[ R^2 = 0.9858 \]

Figure 2-5. Serum IGF-I (ng/mL). Dwarf rats showed IGF-I serum levels significantly decreased when compared to Lewis rats (P<0.0001)
Figure 2-6. Total Bone Mineral Content (BMC) at distal femoral metaphysis (mg/mm). Mean value for dwarf rats was significantly lower than for Lewis rats (P<0.0001)

Figure 2-7. Total Bone Mineral Density (BMD) at distal femoral metaphysis (mg/cm³). A significant decrease was observed in dwarf rats (P<0.0001)
Figure 2-8. Trabecular BMC (mg/mm). A remarkable difference was noted between Lewis and dwarf rats (P<0.0001)

Figure 2-9. Trabecular BMD (mg/cm³). A highly significant difference was observed between Lewis and dwarf rats (P<0.0001)
Figure 2-10. Total Area for Cancellous Bone (mm$^2$). The mean value was significantly lower for dwarf rats compared to Lewis rats (P<0.0001)

Figure 2-11. Trabecular Area (mm$^2$). A significant difference between Lewis and dwarf rats was observed with P<0.001
Figure 2-12. Total BMC at femoral diaphysis (mg/mm). Mean value for dwarf rats was significantly lower than for Lewis rats (P<0.0001).

Figure 2-13. Total BMD at femoral diaphysis (mg/cm³). A significant difference between Lewis and dwarf rats was observed (P<0.0009).
Figure 2-14. Cortical BMC (mg/mm). A significant decrease was observed in dwarf rats compared to Lewis rats (P<0.0001)

Figure 2-15. Cortical BMD (mg/cm³). This was the only pQCT parameter that did not show a significant difference between Lewis and dwarf rats
Figure 2-16. Cortical Area (mm²). Significant decrease in dwarf rats compared to Lewis rats (p<0.0001)

Figure 2-17. Cortical Thickness (mm). Dwarf rats showed a significantly lower mean value than Lewis rats (P<0.0001)
Figure 2-18. Periosteal Circumference (mm). A significant difference ($P<0.0001$) was observed between Lewis and dwarf rats.

Figure 2-19. Endosteal Circumference (mm). Dwarf rats showed a significantly lower mean value compared to Lewis rats ($P<0.0001$).

Figure 2-20. Vertebral Cancellous Bone Volume (%). A significantly lower mean value was observed in dwarf rats (P<0.0001)

Figure 2-21. Vertebral Trabecular Number (#/mm). Dwarf rats showed a significantly lower mean value, with P<0.005
Figure 2-22. Vertebral Trabecular Width (μm). A significant difference between Lewis and dwarf rats was observed, with P<0.001.

Figure 2-23. Vertebral Trabecular Separation (μm). Dwarf rats exhibited a significant increase compared to Lewis rats (P<0.0006).
Figure 2-24. Vertebral Osteoid Surface (%). Dwarf rats did not show a significant difference compared to Lewis rats.

Figure 2-25. Vertebral Osteoblast Surface (%). There was no significant difference between dwarf and Lewis rats.
Figure 2-26. Vertebral Osteoclast Surface (%). There was no significant difference between dwarf and Lewis rats.

Figure 2-27. Vertebral Cancellous Mineral Apposition Rate (μm/d). A significantly lower mean value was observed in dwarf rats compared to Lewis rats. (P<0.0001)
Figure 2-28. Vertebral Mineralizing Surface (%). Dwarf rats showed a significantly decreased mean value compared to Lewis rats (p<0.0001)

Figure 2-29. Vertebral Bone Formation Rate ($10^{-2}\text{um}^3/\text{um}^2/d$). Mean value was significantly decreased in dwarf rats compared to Lewis rats (p<0.0001)
Figure 2-30. Tibial Longitudinal Bone Growth (μm/d). Dwarf rats presented a significantly lower mean value in comparison to Lewis rats (p<0.0001).

Figure 2-31. Proximal tibial metaphyses from Dwarf (A) and Lewis (B) rats. Note the reduced mass of black-stained bone indicative of cancellous osteopenia in the Dwarf rat. Von Kossa/tetrachrome stain, X40. Photos courtesy of Dr. Thomas J.Wronski.
Figure 2-32. Tibial Cancellous Bone Volume (%). Mean value was markedly lower in dwarf rats than in Lewis rats (P<0.0001)

Figure 2-33. Tibial Trabecular Number (#/mm). Dwarf rats showed a lower mean value than Lewis rats, with P<0.0001
Figure 2-34. Tibial Trabecular Width (μm). Dwarf rats exhibited a significantly lower mean value than Lewis rats, with P<0.0001

Figure 2-35. Tibial Trabecular Separation (μm). The mean value for dwarf rats was significantly higher than that for Lewis rats, with P<0.016
Figure 2-36. Total Cortical Bone Tissue Area (mm²). The mean value was markedly lower in dwarf rats than in Lewis rats, with P<0.0001

Figure 2-37. Cortical Bone Area (mm²). Mean value was significantly lower in dwarf rats than in Lewis rats (P<0.0001)
Figure 2-38. Marrow Area (mm²). Dwarf rats showed a lower mean value compared to Lewis rats (P<0.02)

Figure 2-39. Periosteal Perimeter (mm). Dwarf rats presented a significantly lower mean value than Lewis rats (P<0.02)
Figure 2-40. Endocortical Perimeter (mm). Mean value was significantly lower in dwarf rats than in Lewis rats (P<0.04)

Figure 2-41. Cortical Width (μm). The mean value for dwarf rats was significantly lower than that of Lewis rats, with P<0.0001
Figure 2-42. Periosteal Mineralizing Surface (%). Dwarf rats showed a significantly lower mean value than Lewis rats, with P<0.0007

Figure 2-43. Periosteal Mineral Apposition Rate (µm/d). Mean value was significantly lower in dwarf rats than in Lewis rats (P<0.0001)
Figure 2-44. Periosteal Bone Formation Rate ($10^{-2} \mu m^3/\mu m^2/d$). Dwarf rats showed a lower mean value compared to Lewis rats ($P<0.0001$)

Figure 2-45. Endocortical Mineralizing Surface (%). A significantly lower mean value was observed in dwarf rats compared to Lewis rats ($P<0.0015$)
Figure 2-46. Endocortical Mineral Apposition Rate (μm/d). Dwarf rats presented a significantly lower mean value than Lewis rats (P<0.0008).

Figure 2-47. Endocortical Bone Formation Rate (10^-2μm^3/μm^2/d). Dwarf rats presented a significantly lower mean value than Lewis rats (P<0.0001).
Figure 2-48. Images of lumbar vertebral body obtained for area analysis with Image J software. Photo courtesy of Dan Barousse
Figure 2-49. Biomechanical Load (N). Dwarf rats showed a significantly lower mean value compared to Lewis rats (P<0.03)

Figure 2-50. Biomechanical Stress (N*mm² or MPa) There was no significant difference between dwarf and Lewis rats
Figure 2-51. Biomechanical Stiffness (N/mm). No significant difference was observed between dwarf and Lewis rats.
CHAPTER 3
THE BONE ANABOLIC EFFECTS OF PTH TREATMENT IN GH/IGF-I DEFICIENT DWARF RATS AND THEIR BACKGROUND STRAIN, LEWIS RATS.

Introduction

Parathyroid hormone (PTH) is an 84-amino acid polypeptide synthesized by parathyroid gland cells, and highly conserved among mammalian species. It induces bone anabolic effects when administered intermittently at low doses (Dempster et al., 1993, Canalis et al., 2007, Jilka, 2007). Teriparatide [(PTH (1-34))] is the synthetic or recombinant segment of human parathyroid hormone, amino acid sequence 1 through 34, of the complete molecule containing 84 amino acids, and has the same anabolic properties as PTH in humans (Bilezikian and Rubin, 2006) and rats (Mosekilde et al., 1991). The full-length PTH (1-84) is available only in Europe, while the foreshortened aminoterminal form is available and approved in the United States by the Food and Drug Administration (FDA) since November 2002, for the treatment of osteoporosis in postmenopausal women and men at high risk for fractures. In this condition are included patients with a T score determined by dual-energy x-ray absorptiometry (DXA) lower than -3.0, patients with glucocorticoid-induced osteoporosis (GIOP), osteoporotic patients resistant to other available treatments, and patients with previous fragility fractures. The indications for PTH therapy are restricted to these specific conditions mainly because of the high cost of treatment and the need for daily subcutaneous administration (Canalis et al., 2007).

PTH is the most important endocrine regulator in calcium homeostasis in mammals, involving different receptors and multiple signaling mechanisms (Kousteni and Bilezikian, 2008). Similar to other peptide hormones, PTH effects are mediated through a G protein-coupled receptor system in the cells of targeted tissues that
promotes the activation of intracellular events and various biochemical pathways. Three PTH receptors, PTHR1, PTHR2, and PTHR3, have been identified, but the majority of the physiological actions of PTH are mediated by PTHR1 (Molina, 2006b). Although PTHR1 is primarily and abundantly present in osteoblasts, it is also expressed by osteocytes and bone lining cells (Dempster et al., 1993, Swarthout et al., 2002, Poole and Reeve, 2005, Keller and Kneissel, 2005). Intermittent PTH promotes osteoblastogenesis and increases the number of osteoblasts by regulating their proliferation, differentiation and survival (Dempster et al., 1993, Canalis et al., 2007, Jilka, 2007, Jilka et al., 2009, Jilka et al., 2010). PTH stimulates the response of osteocytes to mechanical strain and shear forces, and preserves osteocyte viability through anti-apoptotic actions (Bilezikian and Rubin, 2006, Kousteni and Bilezikian, 2008). Additionally, PTH regulates the production of sclerostin (SOST), an osteocyte-produced antagonist of bone formation, by inhibiting SOST expression in vitro and in vivo (Keller and Kneissel, 2005, Poole and Reeve, 2005). Apparently, the effects on bone resorption are more likely to be indirect, upregulating RANKL (receptor activator of nuclear factor-κB ligand) and the expression of osteoprotegerin (OPG), which activates and inhibits osteoclastogenesis (Huang et al., 2004, Bilezikian and Rubin, 2006, Kousteni and Bilezikian, 2008).

PTH-related protein (PTHrP) is related in function and structure to PTH, sharing 13 amino acids with the amino-terminal of the hormone, and binds to one of the PTH receptors, PTHR1. Therefore, PTHrP can induce the same effects as PTH in bone and kidney. PTH or PTHrP binding to the receptor stimulates the synthesis of cAMP and activation of protein kinase A and the phosphorylation of targeted proteins, that once
activated, lead to the induction of gene transcription. Activation of the receptor can also involve additional signaling pathways, activating phospholipase C. These two pathways regulate some osteoblast functions, although the exact signaling pathway and the in vivo response to PTH are still not completely determined, and may explain whether the hormone has catabolic or anabolic effects in the skeleton (Bilezikian and Rubin, 2006, Canalis et al., 2007). PTH treatment has been evaluated in many different animal models and pathological conditions, such as the ovariectomized rat model for postmenopausal bone loss (Wronski et al., 1993, Wronski and Yen, 1994), fracture healing (Nozaka et al., 2008, Barnes et al., 2008), and in many other genetically modified rodents models, such as IGF-I knockout mice (Bikle et al., 2002), mice with deletion of the genes for insulin receptor substrate 1 and 2 (IRS.1 -/- and IRS.2-/-) (Yamaguchi et al., 2005), and mice with IGF-I receptor null mutation (Wang et al., 2007). In order to evaluate the influence of the components of the ternary IGF-I complex on the response to PTH treatment, Yakar et. al. (2006) generated three types of mutant mice: with deletion of the liver specific IGF-I gene (LID mice), with global deletion of the acid-labile subunit (ALS) gene (ALSKO mice), and with both liver and ALS inactivated genes (LA mice) (Yakar et al., 2006). In general these studies showed that IGF-I is essential for PTH to induce bone anabolic effects. Nevertheless, the mechanisms for the skeletal response to PTH are not completely understood, and the role of IGF-I as a potential mediator for the bone anabolic effects of PTH is still controversial (Jilka, 2007, Bikle, 2008).
Materials and Methods

Animal Models

In the first experiment, dwarf rats expressed significantly decreased IGF-I serum levels compared to their background strain, Lewis rats, which supported their use as a reliable model to reproduce the physiological changes seen in IGF-I deficiency. Therefore, the dwarf and Lewis strains of rats were used to evaluate the skeletal response to PTH treatment. Based on the same criteria observed for the animals from the first experiment, five-weeks-old female dwarf and Lewis rats, obtained from Harlan Laboratories (UK), were kept in a temperature and humidity controlled environment (25°C), and on a 12h light/12h dark cycle. The experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Florida.

Experimental Design

Following the first analysis to establish the dwarf rat as an animal model for our studies, we tested the hypothesis that IGF-I acts as a potential mediator for the bone anabolic effects of PTH. The four groups (N=13, for vehicle-treated dwarf and Lewis rats, and N=7-10 for PTH-treated dwarf and Lewis rats) were maintained in the same nutritional and environmental conditions until 9 weeks of age, when treatment started. They were routinely evaluated and weighed once a week, for monitoring their health status.

Vehicle and PTH Treatment

The rats from the first experiment, treated with vehicle solution (VEH), formed the control groups, for comparison with the PTH-treated dwarf and Lewis groups. Preparation of the vehicle solution was described above for experiment one. The PTH solution was prepared as follows:
1. Heat inactivated serum was used to prepare the PTH stock solution with human synthetic PTH, 1-34 hPTH (Bachem Laboratory, Torrance, CA), according to the respective strain. Serum from Lewis rats was used to prepare the PTH stock solution for Lewis rats, and in the same way, we used serum from dwarf rats for the preparation of the PTH stock solution to treat dwarf rats.

2. The PTH concentration was adjusted to 1 mg/mL, by adding vehicle.

3. The rats were injected, subcutaneously, daily for 2 weeks with hPTH 1-34 at a dose of 50 μg/kg body weight.

**Bone Formation Markers**

We used the same markers as for experiment one, to be consistent with the methodology. All rats received subcutaneous injections of fluorochrome compounds, declomycin and calcein (Sigma Chemical Co., St. Louis, MO), at a dose of 15 mg/kg of body weight, ten and three days prior to euthanasia respectively, in order to label actively forming bone surfaces.

Following these initial steps, the methods are the same as described in Chapter 2 (pages 34-41), including necropsy procedures, tissue processing, data collection and statistical analysis.

**Results**

**Body Weight and Femoral Length**

The evident phenotypic differences between Lewis and dwarf rats remained proportionally the same after treatment with PTH. At the time of euthanasia, the mean body weights between the Lewis and the dwarf rats were significantly different, as detected in the previous experiment, but not between the VEH- and PTH-treated rats, regardless if the rats were in the Lewis (194.5 ± 12.91g vs. 203 ± 13.5g) or dwarf groups (126.5 ± 6.8g vs. 121.9 ± 8.2g), as seen in Figure 3-1.
Femur length values were decreased in dwarf rats, when compared to Lewis rats, but no significant difference was observed between VEH- and PTH-treated dwarf rats (27.28 ± 0.32mm vs. 27.64 ± 0.67mm). PTH-treated Lewis rats showed a slight, but significant increase in mean femur length compared to VEH-treated Lewis rats (32.13 ± 0.63mm vs. 31.19± 0.51mm, respectively, P<0.0026), as seen in Figure 3-2.

**IGF-I Serum Levels**

Serum levels of IGF-I were markedly lower in VEH-treated dwarf rats compared with VEH-treated Lewis rats (353.36 ± 153.15 ng/mL vs. 845.22 ± 169.45 ng/mL, P<0.0001), as seen in experiment 1. Regardless of treatment, there were no significant differences in IGF-I serum levels in either PTH-treated Lewis rats compared to their VEH-treated controls (755.00 ± 202.00 ng/mL vs. 845.22 ± 169.45 ng/mL), or in PTH-treated dwarf rats compared to their VEH-treated controls (265.53 ± 95.28 ng/mL vs. 353.36 ± 153.15 ng/mL), as shown in Figure 3-3.

**Peripheral Quantitative Computerized Tomography**

The pQCT analyses of the distal femoral metaphysis revealed that cancellous bone structural parameters (total BMC, total BMD, trabecular BMC, and trabecular BMD) were significantly higher in PTH-treated dwarf and Lewis rats, when compared to vehicle-treated rats, as shown in Figures 3-4 to 3-9. The trabecular density measurements demonstrated a significant interaction between group and treatment. Post hoc analysis found that PTH-treated dwarf rats had a greater increase in trabecular BMD when compared to PTH-treated Lewis rats, similar to the results obtained for longitudinal bone growth (see below).

When considering cortical bone parameters (Figures 3-10 to 3-17), almost all values obtained at the femoral shafts for total BMC, total BMD, cortical BMC, cortical
area, cortical thickness, periosteal and endocortical circumferences, did not show any
PTH treatment effects in either group, with the exception of cortical BMD, which showed
a slight, but significant decrease in PTH-treated dwarf rats (P=0.0013).

Histomorphometric Findings

Cancellous bone measurements in lumbar vertebrae

PTH significantly increased vertebral cancellous bone volume in both dwarf
(25.1±6.7% vs. 15.9±4.4%, P=0.0016) and Lewis rats (37.0±6.4% vs. 26.4±6.4%,
P<0.0001) when compared to vehicle-treated rats, as shown in Figure 3-18, and this
effect was associated with increased trabecular width in PTH-treated dwarf (P=0.0229)
and Lewis (P=0.0320) rats. There was a slight, but nonsignificant increase in trabecular
number between PTH- and vehicle-treated rats in both groups, and a strong trend for
decreased trabecular separation in PTH-treated dwarf (P=0.0567) and Lewis rats
(P=0.063), as seen in Figures 3-19 to 3-21.

PTH treatment increased cancellous bone surface parameters, osteoid and
osteoblast surfaces, to the same extent, in both dwarf and Lewis rats. There was no
significant difference in the percentage increase between the two strains, but a highly
significant increase when PTH-treated dwarf and Lewis rats were compared to their
vehicle-treated controls, as seen in Figures 3-22 and 3-23. The osteoclast surface was
significantly increased only in PTH-treated dwarf rats (Figure 3-24).

When analyzing bone kinetic variables, we found that PTH-treated dwarf rats
exhibited 7- and 13-fold increases in mineralizing surface and bone formation rate,
respectively, compared to vehicle-treated dwarf rats. PTH-treated Lewis rats showed 3-
and 4-fold increases in mineralizing surface and bone formation rate compared to
vehicle-treated Lewis rats, as seen in Figures 3-25 and 3-26. Cancellous bone mineral
apposition rate, an index of osteoblast activity, was increased in PTH-treated dwarf rats (1.25±0.14 μm vs. 0.88±0.09 μm, P<0.0001) and in PTH-treated Lewis rats (1.35±0.08 μm vs. 1.07±0.12 μm, P<0.0001) compared to their respective vehicle-treated groups (Figure 3-27).

**Cancellous bone measurements in proximal tibiae**

When compared to vehicle treatment, tibial longitudinal bone growth was significantly higher in both PTH-treated groups of dwarf rats (27.29 ± 8.67 μm/d vs. 72.79 ± 6.11 μm/d, P<0.0001) and Lewis rats (70.88 ± 5.55 μm/d vs. 82.27 ± 12.66 μm/d, P=0.0321), as seen in Figure 3-28. The effect of PTH treatment on tibial cancellous bone structure is even more noticeable in the histologic images from vehicle- and PTH-treated dwarf rats in Figure 3-29. Among the cancellous bone structural values for the proximal tibia, we observed the same positive effect of PTH on tibial cancellous bone volume, in both dwarf (2.78 ± 1.83% vs. 8.78 ± 2.78%, P=0.0016) and Lewis rats (19.36 ± 2.75% vs. 24.58 ± 4.33%, P<0.0023), (Figure 3-30). The effect of PTH on bone volume was associated with increased trabecular number in dwarf rats (1.52 ± 0.93 #/mm vs. 3.48 ± 0.88 #/mm, P=0.0001) but not in Lewis rats (6.19 ± 0.90 #/mm vs. 6.82 ± 0.30 #/mm, P=0.3484) (Figure 3-31), and with increased trabecular width only in dwarf rats (21.35 ± 3.0 μm vs. 29.96 ± 2.21 μm, P=0.0183) (Figure 3-32).

In addition, no significant differences were observed in trabecular separation in dwarf and Lewis rats treated with PTH (Figure 3-33), although a strong trend for an increase in this parameter was observed in the former animals.

**Cortical bone measurements in tibial diaphysis**

The effects of PTH treatment on cortical bone were observed in the analysis of the left tibial diaphyses, as shown in Figures 3-34 to 3-39. The total cortical bone tissue
area, cortical area, marrow area, periosteal perimeter, and endocortical perimeter showed no significant effects of PTH treatment either in dwarf or Lewis rats. However, cortical width was slightly, but significantly decreased in PTH-treated dwarf rats (P=0.0116).

All the dynamic measurements at the tibial diaphysis, periosteal and endocortical mineralizing surface, mineral apposition rate, and bone formation rate were markedly increased in PTH-treated dwarf rats. PTH-treated Lewis rats showed significantly increased periosteal mineral apposition and bone formation rates (P=0.0072 and P=0.0026, respectively), as seen in Figures 3-40 to 3-45.

**Biomechanical Testing in Lumbar Vertebral Body**

The biomechanical parameter of stress in the lumbar vertebrae of PTH-treated dwarf rats was increased when compared to vehicle-treated dwarf rats (P=0.0115). The same increase was not observed in PTH-treated Lewis rats (P=0.0818). PTH treatment did not cause a significant increase in dwarf rats in the other parameters, load and stiffness, but PTH-treated Lewis rats showed a significant increase in load, with P=0.0004, as seen in Figures 3-46 to 3-48.

**Discussion**

Our findings indicate that PTH stimulates bone formation and increases cancellous bone mass in dwarf rats with low serum levels of IGF-I. These results may be considered to contrast with the majority of studies that postulate IGF-I as an essential mediator for the bone anabolic effects of PTH, since initial *in vitro* experiments indicated that PTH stimulates IGF-I release from calvarial osteoblasts (Linkhart et al., 1989), and that IGF-I antibody inhibits PTH-induced stimulation of collagen synthesis in bone cultures (Canalis et al., 1989). Furthermore, PTH has been reported to participate in
the regulation of local and serum IGF-I levels and to stimulate IGF-I synthesis by osteoblasts (Dempster et al., 1993, He et al., 2006). Bone mass and bone matrix IGF-I increased in rats treated with PTH, but there was no effect on serum IGF-I levels, suggesting that IGF-I acts as a local mediator of PTH anabolic effects (Pfeilschifter et al., 1995). In a study to detect the early effects of short term PTH administration, decreased IGF-I serum concentrations were observed throughout the study, which contrasted with previously published data (Toromanoff et al., 1998). Knockout mice with global deletion of the acid-labile subunit (ALS), named ALSKO, provided evidence that the IGF-I ternary complex is important for bone remodeling and for the anabolic response to PTH treatment (Yakar et al., 2006).

Dwarf rats are also deficient in GH, and the anabolic effects of PTH (1-34) may depend on GH, as suggested by studies with hypophysectomized rats (Hock and Fonseca, 1990). However, PTH increased bone mass by stimulating bone formation in aged female rats, regardless of GH treatment (Gunness, 1995). Despite low serum IGF-I and GH levels, we found that PTH treatment increased cancellous bone mass and stimulated both cancellous and cortical bone formation in dwarf rats.

The pQCT data from the distal femoral metaphysis revealed increased mineral density and mineral content of cancellous bone with PTH treatment in both Lewis and dwarf rats; in fact, dwarf rats showed a somewhat greater increase in trabecular bone density compared to Lewis rats, with a significant group-treatment interaction. At the femoral shaft, only the cortical BMD was significantly higher in the PTH-treated dwarf rats. In most studies, the anabolic effects of PTH are more pronounced in cancellous
bone than in cortical bone (Dempster et al., 1993). Our results from the distal femoral metaphysis and femoral shaft are in agreement with this finding.

PTH increases the number of osteoblasts and their activity, which results in thicker trabeculae and improves trabecular connectivity, restoring bone microarchitecture (Hodsman et al., 2005, Bilezikian and Rubin, 2006). The bone measurements in the lumbar vertebrae and proximal tibiae of PTH-treated dwarf and Lewis rats, in accordance with previous studies, showed significant increases in bone volume, associated with increased trabecular width. In addition to the PTH-induced increase in bone density and mineral content observed with pQCT, biomechanical testing revealed that the lumbar vertebrae in PTH-treated dwarf rats did not show a significant increase in load and stiffness, but the observed increase in the parameter stress (N*mm²), which includes the area in its calculation, seems more relevant in this analysis, as the vertebrae in dwarf rats were very small. Nevertheless, vertebral load, which was significantly increased in PTH-treated Lewis rats, would undoubtedly have also been increased in PTH-treated dwarf rats with a longer treatment period.

In contrast to antiresorptive agents used in the treatment of osteoporosis, PTH has the capability of stimulating bone turnover (Bilezikian and Rubin, 2006, Canalis et al., 2007, Kousteni and Bilezikian, 2008). Consistent with this concept, osteoclast surface, an index of bone resorption, was significantly increased in PTH-treated dwarf rats. Furthermore, mineralizing surface and bone formation rate were notably increased in PTH-treated dwarf and Lewis rats, compared to their vehicle-treated controls. The anabolic effect of PTH in dwarf rats may have been superior to that observed in PTH-treated Lewis rats. Moreover, cancellous bone mineral apposition rate, an index of
osteoblast activity, was increased in PTH-treated dwarf and Lewis rats compared to their respective control groups. Therefore, PTH stimulated both cancellous bone resorption and formation in dwarf and Lewis rats. Regardless of IGF-I status, the increment in bone formation must have exceeded the increment in bone resorption for the observed PTH-induced gain in bone mass to occur.

IGF-I is decreased, but not abolished in the dwarf rat, which is different from mice with global deletion of the \textit{IGF-I} gene. This may explain why PTH fails to induce a bone anabolic response in IGF-I knockout mice, but has a strong anabolic effect in dwarf rats. Different responses to PTH can occur, as mixed genetic background can affect bone acquisition and response to PTH treatment (Yakar et al., 2006), and not all patients and not all bones respond to intermittent treatment with PTH in the same manner (Bikle, 2008). Human patients with \textit{IGF-I} gene deletion have been identified, but cases are rare (Yakar et al., 2006), while IGF-I deficiency is present in several osteopenic conditions. Different responses to PTH treatment have also been observed in healthy inbred strains of mice with differences in skeletal IGF-I synthesis (Bilezikian and Rubin, 2006). Yet, in addition to the GH and IGF-I skeletal effects, the participation of the six IGF-I binding proteins must also be considered. The different cellular signaling pathways that are concomitantly influenced by the GH/IGF-I axis and PTH, suggest that several levels of regulation might be involved in the role of IGF-I as a mediator for the bone anabolic effects of PTH \textit{in vivo}.(Pereira and Canalis, 1999). Nevertheless, our findings indicate that PTH has a strong stimulatory effect on bone formation and augments bone mass even in the presence of low circulating levels of IGF-I.

Conclusions

The results addressed Specific Aim 2, and the major findings were:
1. PTH did not affect body weight or serum IGF-I levels in either Lewis or dwarf rats.

2. PTH induced highly significant anabolic effects in vertebral and tibial cancellous bone in dwarf rats, and stimulated both cancellous and cortical bone formation, despite low circulating levels of IGF-I.
Figure 3-1. Body weight (g). Significant difference between Lewis and dwarf rats (P<0.0001), but not between VEH- and PTH-treated rats in either the Lewis or dwarf groups.

Figure 3-2. Femur Length (mm). PTH treatment slightly increased mean femur length in Lewis rats (P=0.0026), but no difference was observed between VEH- and PTH-treated dwarf rats.
Figure 3-3. Serum IGF-I (ng/mL). A significant difference was observed between Lewis and dwarf rats (P<0.0001), but not between VEH- and PTH-treated rats in either group.

Figure 3-4. Total Bone Mineral Content (BMC, mg/mm) in distal femoral metaphysis. Significant increases were observed in PTH-treated rats from both groups (P<0.0001).
Figure 3-5. Total Bone Mineral Density (BMD) in the distal femoral metaphysis (mg/cm$^3$). Significant increases were observed in dwarf and Lewis rats treated with PTH (P<0.0001)

Figure 3-6. Trabecular BMC (mg/mm). Significant increases were observed in both Lewis and dwarf rats treated with PTH (P<0.0001)
Figure 3-7. Trabecular BMD (mg/cm³). Highly significant increases were observed in dwarf (P<0.0001) and Lewis (P=0.0022) rats treated with PTH.

Figure 3-8. Total Area for distal femoral metaphysis (mm²). A significant increase was observed in PTH-treated dwarf rats (P=0.0202).
Figure 3-9. Trabecular Area (mm²). A significant increase was observed in PTH-treated Lewis rats (P=0.0169).

Figure 3-10. Total BMC for femoral diaphysis (mg/mm). There were no significant differences in the PTH-treated rats, dwarf or Lewis, when compared to their respective vehicle-treated controls.
Figure 3-11. Total BMD for femoral diaphysis (mg/cm³). There were no significant differences between PTH- and vehicle-treated groups in dwarf and Lewis rats.

Figure 3-12. Cortical BMC (mg/mm). No significant differences were found between the PTH- and vehicle-treated groups for both dwarf and Lewis rats.
Figure 3-13. Cortical BMD (mg/cm³). There was a significant difference between PTH- and vehicle-treated dwarf rats (P=0.0013), but not between these groups of Lewis rats.

Figure 3-14. Cortical Area (mm²). No significant differences were observed with PTH treatment in dwarf and Lewis rats.
Figure 3-15. Cortical Thickness (mm). There were no significant differences between PTH- and vehicle-treated groups for dwarf and Lewis rats.

Figure 3-16. Periosteal Circumference (mm). The values were almost identical between PTH- and vehicle-treated groups for dwarf and Lewis rats.
Figure 3-17. Endosteal Circumference (mm). No significant differences were observed with PTH treatment in both groups

Figure 3-18. Vertebral Cancellous Bone Volume (%). There were significant increases in PTH-treated dwarf (P=0.0016) and Lewis (P<0.0001) rats when compared to their vehicle control groups
Figure 3-19. Vertebral Trabecular Number (#/mm). There was a slight, but nonsignificant increase between PTH- and vehicle-treated rats in both groups.

Figure 3-20. Vertebral Trabecular Width (μm). There was a significant increase in PTH-treated dwarf (P=0.0229) and Lewis (P=0.0320) rats, when compared to their vehicle control groups.
Figure 3-21. Vertebral Trabecular Separation (μm). Strong trends for decreases were observed in dwarf (P=0.00567) and Lewis (P=0.0603) rats treated with PTH.

Figure 3-22. Vertebral Osteoid Surface (%). Highly significant increases were noted in dwarf (P<0.0001) and Lewis (P=0.0027) rats treated with PTH.
Figure 3-23. Vertebral Osteoblast Surface (%). Markedly significant increases were noted in dwarf (P<0.0001) and Lewis (P=0.0011) rats treated with PTH.

Figure 3-24. Vertebral Osteoclast Surface (%). A highly significant increase was noted only in dwarf rats treated with PTH (P=0.0125).
Figure 3-25. Vertebral Mineralizing Surface (%). Highly significant increases were noted in dwarf and Lewis rats treated with PTH (P<0.0001)

Figure 3-26. Vertebral Bone Formation Rate (10^{-2} \mu m^3/\mu m^2/d). Markedly significant increases were noted in dwarf and Lewis rats treated with PTH (P<0.0001)
Figure 3-27. Vertebral Cancellous Mineral Apposition Rate (μm/d). Significant increases were observed in PTH-treated dwarf and Lewis rats (P<0.0001)

Figure 3-28. Longitudinal Bone Growth (μm/d). Significant increases were observed in PTH-treated dwarf (P<0.0001) and Lewis (P=0.0321) rats
Figure 3-29. Proximal tibial metaphyses from vehicle-treated dwarf (A) and PTH-treated dwarf (B) rats. Note the reduced mass of black-stained bone indicative of cancellous osteopenia in the vehicle-treated dwarf rat, compared to the increased number of thicker trabeculae in the PTH-treated dwarf rat. Von Kossa/tetrachrome stain, X40. Photos courtesy of Dr. Thomas J. Wronski

Figure 3-30. Tibial Cancellous Bone Volume (%). Significant increases were observed in both dwarf (P=0.0016) and Lewis (P=0.0023) rats treated with PTH.
Figure 3-31. Tibial Trabecular Number (#/mm). A significant increase was observed only in PTH-treated dwarf rats ($P=0.0001$).

Figure 3-32. Trabecular Width (μm). A significant increase was observed in PTH-treated dwarf rats ($P=0.0183$), but not in PTH-treated Lewis rats.
Figure 3-33. Tibial Trabecular Separation (µm). No significant differences were observed in PTH-treated dwarf and Lewis rats.

Figure 3-34. Total Cortical Bone Tissue Area in Tibial Diaphysis (mm²). No significant differences were observed with PTH treatment in dwarf and Lewis rats.
Figure 3-35. Cortical Bone Area in Tibial Diaphysis (mm$^2$). No significant differences were observed with PTH treatment in dwarf and Lewis rats.

Figure 3-36. Bone Marrow Area (mm$^2$). There were no significant differences with PTH treatment in both groups.
Figure 3-37. Periosteal Perimeter (mm). No significant differences were observed with PTH treatment in both dwarf and Lewis rats.

Figure 3-38. Endocortical Perimeter (mm). No significant differences were observed with PTH treatment in both dwarf and Lewis rats.
Figure 3-39. Cortical Width (µm). Cortical width decreased in PTH-treated dwarf rats (P=0.0116), but not in PTH-treated Lewis rats.

Figure 3-40. Periosteal Mineralizing Surface (%). A significant increase was observed in PTH-treated dwarf rats (P<0.0001).
Figure 3-41. Periosteal Mineral Apposition Rate (μm/d). PTH treatment induced significant increases in both dwarf (P<0.0001) and Lewis (P=0.0072) rats.

Figure 3-42. Periosteal Bone Formation Rate (10^{-2} μm^3/μm^2/d). Significant increases in PTH-treated dwarf (P<0.0001) and Lewis (P=0.0026) rats were observed.
Figure 3-43. Endocortical Mineralizing Surface(%). A significant increase was observed in PTH-treated dwarf rats (P=0.0022)

Figure 3-44. Endocortical Mineral Apposition Rate (um/d). A significant increase was observed in dwarf rats with PTH treatment (P=0.0006)
Figure 3-45. Endocortical Bone Formation Rate ($10^{-2}\ \mu \text{m}^3/\mu \text{m}^2/d$). A significant increase was observed in PTH-treated dwarf rats ($P=0.0002$)

Figure 3-46. Biomechanical Load (N). A significant increase was observed only in PTH-treated Lewis rats ($P=0.0004$)
Figure 3-47. Biomechanical Stress (N*mm$^2$ or MPa). A significant increase was observed only in PTH-treated dwarf rats (P=0.0115)

Figure 3-48. Biomechanical Stiffness (N/mm). No significant differences were observed with PTH treatment in both dwarf and Lewis rats
CHAPTER 4
COMPARISON OF PGE$_2$ TREATMENT IN GH/IGF-I DEFICIENT DWARF RATS AND THEIR BACKGROUND STRAIN, LEWIS RATS.

Introduction

Prostaglandins (PGs) are critical regulators of both skeletal physiologic and pathologic responses, acting in local tissues as paracrine-autocrine factors (Raiz and Lorenzo, 2006). The synthesis of PGs by bone cells is regulated by local factors, as well as systemic hormones that participate in bone metabolism, including PTH and vitamin D$_3$ (Pilbeam et al., 2008). Prostaglandins are considered dual regulators of bone metabolism, stimulating both bone formation and bone resorption \textit{in vivo} (Lin et al., 1995), but with a positive balance in favor of bone formation (Harada et al., 1995, Jee and Ma, 1997). Prostaglandins are synthesized by many cells in the skeleton, and prostaglandin E$_2$ (PGE$_2$) is abundant in bone cells, acting mainly through receptor EP4 to stimulate bone formation, as demonstrated \textit{in vitro} and \textit{in vivo} (Ke et al., 2006, Aguirre et al., 2007, Downey et al., 2009). \textit{In vitro} studies showed both stimulatory and inhibitory effects of PGE$_2$ on bone formation in cell and organ culture, and IGF-I was also considered as a mediator for the effects of PGE$_2$ on osteoblasts (Pilbeam et al., 2008). Machwate \textit{et al.} (2001) examined the effect of an EP4 specific antagonist, EP$_4$A, on bone formation induced by PGE$_2$ in young rats. They found that EP$_4$A suppressed the increase in bone mass induced by PGE$_2$. This effect is accompanied by a reduction in the extent of calcein-labeled surface and trabecular number, suggesting that EP$_4$ is the main receptor through which PGE$_2$ induces bone formation in rats (Machwate \textit{et al.}, 2001).

The bone anabolic effect of PGE$_2$ has been compared to the effects of PTH (Harada et al., 1995). \textit{In vivo}, systemic injection of PGE$_2$ in rats increases both cortical
and cancellous bone formation, and produces substantial increases in bone mass, similar to the effects of PTH (Li et al., 1992). Like PGE₂, PTH also increases the production of IGF-I by osteoblasts in vitro (McCarthy et al., 1991), and the anabolic effects of PTH may be mediated by IGF-I (Canalis et al., 1989). Taking these findings into consideration, we decided to evaluate the bone anabolic effects of PGE₂ in IGF-I deficient dwarf rats, to determine whether low serum levels of IGF-I affect the ability of PGE₂ to stimulate bone formation and augment bone mass.

**Materials and Methods**

**Animal Models**

The same groups of vehicle-treated dwarf and Lewis rats, analyzed in the first experiment, were used as controls, for evaluation of the effects of PGE₂ on bone. IGF-I serum levels in dwarf rats were significantly lower compared to their background strain, Lewis rats, reproducing the physiological changes seen in IGF-I deficiency, which validates their use as a reliable model. Therefore, the dwarf and Lewis strains of rats were used to evaluate the effects of PGE₂ treatment on bone mass and turnover. Based on the same criteria for animals from the first and second experiments, five week old female dwarf and Lewis rats, obtained from Harlan Laboratories (UK), were kept in a temperature and humidity controlled environment (25° C), and on a 12h light/12h dark cycle. The experiment was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida.

**Experimental Design**

Following the first analysis to establish the dwarf rat as an animal model for our studies, we tested the hypothesis that IGF-I acts as a potential mediator for the bone anabolic effects of PGE₂. The four groups (N=13, for vehicle-treated dwarf and Lewis
rats, and N=7 for PGE$_2$-treated dwarf and Lewis rats) were maintained in the same nutritional and environmental conditions until 9 weeks of age, when treatment started. All rats were routinely examined and weighed once a week, for monitoring their health status.

**Vehicle and PGE$_2$ Treatment**

The rats from the first experiment, treated with vehicle solution (VEH), formed the control groups, for comparison with the PGE$_2$-treated dwarf and Lewis groups. The vehicle solution was already described for experiment one. The PGE$_2$ solution was prepared as follows:

1. PGE$_2$ (Cayman Chemical Co., Ann Arbor, MI) was supplied as a crystalline solid and the stock solution (15mg/mL) was prepared by dissolving 45 mg of PGE$_2$ in 3 mL of 100% ethanol.

2. For the injections, 0.5 mL of the stock solution was then diluted with sterilized distilled water, obtaining a final concentration of 3 mg/mL.

   The rats were injected, subcutaneously, daily for 2 weeks with PGE$_2$ at a dose of 3 mg/kg body weight.

**Bone Formation Markers**

We used the same fluorochrome markers as described for experiment one. All rats received subcutaneous injections of the fluorochrome compounds, declomycin and calcein (Sigma Chemical Co., St. Louis, MO), at a dose of 15 mg/kg body weight, ten and three days prior to euthanasia respectively, in order to label actively forming bone surfaces. Following these initial steps, the methods are the same as described in Chapter 2 (pages 34-41), including necropsy procedures, tissue processing, data collection, and statistical analysis.
Results

Body Weight

The evident phenotypic differences between Lewis and dwarf rats remained proportionally the same. At the time of euthanasia, the mean body weights for the Lewis and dwarf rats were significantly different, as already observed in the previous experiments. PGE₂-treated Lewis rats, however, weighed significantly less than vehicle-treated rats Lewis rats (194.54 ± 12.90g vs. 167.28± 9.75g, respectively, \( P<0.0001 \)), but this was not weight loss due to PGE₂ treatment. Rather, the PGE₂-treated Lewis rats weighed less than the vehicle-treated Lewis rats at the beginning of treatment, and this difference in body weight was maintained during the 2 week treatment period. No significant difference in body weight was observed in dwarf rats treated with PGE₂ or vehicle, as seen in Figure 4-1.

IGF-I Serum Levels

Serum levels of IGF-I were markedly lower in dwarf rats compared with Lewis rats, as seen in experiment 1. PGE₂ did not increase serum IGF-I levels in either Lewis or dwarf rats. In fact, PGE₂-treated Lewis rats even showed significantly lower IGF-I serum levels than their vehicle-treated controls (Figure 4-2).

Peripheral Quantitative Computerized Tomography

The pQCT analyses of the distal femoral metaphysis (Figures 4-3 to 4-8) revealed that cancellous bone structural parameters, such as trabecular BMC and trabecular BMD, were significantly higher in PGE₂-treated dwarf rats, when compared to vehicle-treated dwarf rats (\( P=0.0251 \) and \( P=0.0011 \)). Total BMD exhibited a significant decrease only in PGE₂-treated dwarf rats (\( P<0.0001 \)). Total BMC, total area, and
trabecular area showed no significant difference with PGE$_2$ treatment in dwarf and Lewis rats when compared to their vehicle-treated controls.

When considering cortical bone (Figures 4-9 to 4-16), however, almost all the parameters measured at the femoral shafts showed significantly lower values in the PGE$_2$-treated groups. Total BMC and cortical BMD had significantly lower values in the PGE$_2$-treated groups, dwarf and Lewis, compared to their controls. Total BMD, cortical BMC, and cortical area exhibited significant lower values in PGE$_2$-treated dwarf rats. Endocortical circumference and periosteal circumference presented significant lower values only in PGE$_2$-treated Lewis rats. Cortical thickness did not show significant differences in PGE$_2$-treated dwarf and Lewis rats, when compared to their respective vehicle-treated controls.

**Histomorphometric Findings**

**Cancellous bone measurements in the lumbar vertebrae**

When comparing the effects of PGE$_2$ and vehicle treatment on vertebral cancellous bone volume, there were no significant increases in Lewis and dwarf rats treated with PGE$_2$, although a trend was evident, as shown in Figure 4-17. However, PGE$_2$ treatment induced a significant increase in trabecular number, associated with decreased trabecular separation in dwarf and Lewis rats compared to their controls (Figures 4-18 and 4-20), but no significant changes occurred in trabecular width (Figure 4-19).

When analyzing cancellous bone surface parameters, PGE$_2$ treatment did not induce any significant difference in the osteoid, osteoblast, and osteoclast surfaces in dwarf and Lewis rats, as seen in Figures 4-21 to 4-23.
Considering bone kinetic variables (Figures 4-24 to 4-26), PGE$_2$-treated Lewis rats exhibited significantly increased mean values for vertebral cancellous bone mineralizing surface compared to vehicle-treated Lewis rats. PGE$_2$-treated dwarf rats showed only a trend for increased vertebral cancellous bone mineralizing surface and a decreased cancellous bone mineral apposition rate. The mean values for vertebral bone formation rate did not show significant differences in PGE$_2$-treated dwarf and Lewis rats compared to their vehicle-treated controls.

**Cancellous bone measurements in the proximal tibiae**

Tibial longitudinal bone growth was significantly higher in PGE$_2$-treated dwarf rats compared to vehicle-treated dwarf rats (27.29 ± 8.67 µm/d vs. 72.79 ± 6.11 µm/d, $P<0.0001$) whereas PGE$_2$-treated Lewis rats (70.88 ± 5.55 µm/d vs. 82.27 ± 12.66 µm/d, $P=0.0881$) only exhibited a trend for increased longitudinal bone growth, as seen in Figure 4-27. The effect of PGE$_2$ treatment on tibial cancellous bone structure is even more noticeable in histologic images from vehicle- and PGE$_2$-treated dwarf rats in Figure 4-28.

Regarding cancellous bone structural parameters in the proximal tibia, we observed an increase in the tibial cancellous bone volume in dwarf rats, as a response to PGE$_2$ treatment, but not in Lewis rats, as seen in Figure 4-29. The PGE$_2$ effect on bone volume was associated with increased trabecular number only in dwarf rats, but not in Lewis rats, when compared to vehicle-treated controls (Figures 4-30). Trabecular width, however, was decreased in PGE$_2$-treated Lewis rats, but showed no significant difference in dwarf rats in response to PGE$_2$ treatment (Figure 4-31). Tibial trabecular separation (Figure 4-32) was not significantly different in PGE$_2$-treated dwarf and Lewis
rats compared to their vehicle-treated controls, although a strong trend for an increase in this variable was observed in PGE$_2$-treated dwarf rats.

**Cortical bone measurements in the tibial diaphysis**

When analyzing the effects of PGE$_2$ treatment on bone structural parameters in the left tibial diaphyses (Figures 4-33 to 4-38), there was no significant change in the total cortical bone tissue area in both PGE$_2$-treated dwarf and Lewis rats compared to vehicle-treated rats, but the cortical bone area and width showed significantly lower mean values in Lewis rats treated with PGE$_2$. No differences in these parameters were observed in PGE$_2$-treated dwarf rats compared to their vehicle-treated controls. However, PGE$_2$-treated dwarf rats exhibited an increase in marrow area and periosteal perimeter.

Regarding periosteal dynamic measurements in the tibial diaphysis (Figures 4-39 to 4-41), only the periosteal mineral apposition rate was increased in response to PGE$_2$ treatment in dwarf rats. Periosteal mineralizing surface and bone formation rate did not show any difference due to PGE$_2$ treatment in the two strains. At the endocortical surface (Figures 4-42 to 4-44), no effect of PGE$_2$ treatment was observed in mineralizing surface in dwarf and Lewis rats compared to their vehicle-treated controls. PGE$_2$ treatment increased endocortical mineral apposition rate and bone formation rate only in dwarf rats, but not in PGE$_2$-treated Lewis rats.

**Biomechanical Testing in Lumbar Vertebral Body**

The biomechanical analysis revealed an increase in the stress parameter only in PGE$_2$-treated Lewis rats compared to vehicle-treated Lewis rats. No significant differences were detected for load and stiffness parameters in dwarf and Lewis rats.
treated with PGE$_2$, compared to their respective vehicle-treated controls, as seen in Figures 4-45 to 4-47.

**Discussion**

Our results indicate that PGE$_2$ treatment augments bone mass in dwarf rats, mainly in cancellous bone, despite lower serum IGF-I levels. This finding is somewhat surprising in that other studies have suggested that IGF-I or the IGF-I receptor may mediate the stimulatory effects of PGE$_2$ on bone formation (Harada et al., 1995, McCarthy et al., 1991, Raisz et al., 1993a). However, our findings show that low serum levels of IGF-I are adequate to support a PGE$_2$-induced increase in cancellous bone mass.

PGE$_2$-treated rats Lewis rats did not gain as much weight as PGE$_2$-treated rats. This finding may be related to the gastrointestinal side effects that occur during PGE$_2$ therapy (Jee and Ma, 1997). In a study with aged rats, Cui (2001) also observed body weight loss due to diarrhea and reduced activity that persisted 2 to 4 hours after injection (Cui et al., 2001). The weight loss, however, did not affect bone growth in our young rats, as our results showed a significant increase in tibial longitudinal bone growth in the two strains treated with PGE$_2$.

The pQCT data obtained in our study revealed a positive anabolic effect of PGE$_2$ treatment on metaphyseal cancellous bone, with increased trabecular BMC and BMD, but the same response to PGE$_2$ treatment was not observed in cortical bone, where almost all of the parameters presented lower values. Our results diverge from those observed in another *in vivo* study that reported increased cortical bone mass in the tibial shaft, using the same PGE$_2$ dose of 3mg/kg, but for a longer treatment period (30 days) in aged rats (Cui et al., 2001). The weak response to PGE$_2$ treatment in cortical bone...
was also noticed in the histomorphometric analysis we performed in the tibial diaphysis, where the cortical area and the cortical width were decreased (PGE$_2$-treated Lewis rats) or did not show any significant difference (PGE$_2$-treated dwarf rats). The lack of a strong anabolic effect of PGE$_2$ on cortical bone in the current study may be due to the relatively short treatment period (14 days) or the relatively young age of the dwarf and Lewis rats at the beginning of treatment (9 weeks).

Ito (1993) observed differences in the response to PGE$_2$ treatment depending upon cancellous bone sites (proximal and distal tibial metaphysis) and in a dose-dependent manner (Ito et al., 1993). We noticed that the values for cancellous bone volume in the lumbar vertebrae were not increased in PGE$_2$-treated dwarf rats, whereas in the proximal tibial metaphysis, cancellous bone volume was significantly increased in these animals. This finding may be a consequence of a more rapid response to PGE$_2$ treatment in the long bone metaphysis, which has a higher rate of cancellous bone turnover compared to the lumbar vertebral body.

Jee (1997) proposed that the amount of bone formation was higher in the tibial shaft than in cancellous bone of the distal tibial metaphysis, followed by the distal femoral metaphysis, proximal tibial metaphysis and lumbar vertebral body, in decreasing sequence (Jee and Ma, 1997). Although PGE$_2$ has been reported to stimulate bone formation at the periosteal and endocortical surfaces (Raiz and Lorenzo, 2006), we found no significant differences for periosteal mineralizing surface and bone formation rate in Lewis rats treated with PGE$_2$. Only the mineral apposition rate, indicative of osteoblastic activity, was significantly increased by PGE$_2$ treatment in dwarf rats. In addition, the biomechanical analysis of the lumbar vertebral body showed a
significant increase only in the parameter stress, and exclusively in Lewis rats that received PGE₂ treatment. As mentioned above, the treatment period may not have been of sufficient duration for PGE₂ to induce biomechanical changes and fully stimulate cortical bone formation. Nevertheless, our findings demonstrate that lower levels of systemic IGF-I do not affect the bone anabolic response to PGE₂ treatment.

Conclusions

1. PGE₂ treatment did not increase body weight or serum IGF-I levels in either Lewis or dwarf rats.

2. PGE₂-treated rats exhibited significantly higher bone mass, mostly cancellous bone, whereas cortical bone mass was significantly lower.

3. Most importantly, PGE₂ induced significant anabolic effects, mainly in cancellous bone of dwarf rats, despite low circulating levels of IGF-I.
Figure 4-1. Body Weight (g). There was a significant decrease in the body weight of PGE$_2$-treated Lewis rats ($P<0.0001$), but not in PGE$_2$ treated dwarf rats.

Figure 4-2. IGF-I Serum Levels (ng/mL). There was a significant difference between vehicle-treated Lewis and dwarf rats ($P=0.0115$). PGE$_2$-treated Lewis rats showed significantly lower values compared to vehicle-treated Lewis rats ($P=0.0228$).
Figure 4-3. Total Bone Mineral Content (BMC) (mg/mm) in the distal femoral metaphysis. There were no significant differences with PGE2 treatment in dwarf and Lewis rats.

Figure 4-4. Total Bone Mineral Density (BMD) (mg/cm³). A significant decrease was observed in PGE2-treated dwarf rats (P<0.0001), but not in Lewis rats treated with PGE2.
Figure 4-5. Trabecular BMC (mg/mm). There was a significant increase in PGE$_2$-treated dwarf rats (P=0.0251), but not in PGE$_2$-treated Lewis rats.

Figure 4-6. Trabecular BMD (mg/cm$^3$). A significant increase was observed in PGE$_2$-treated dwarf rats (P=0.0011), but not in Lewis rats treated with PGE$_2$.
Figure 4-7. Total Area for Cancellous Bone (mm²). No significant difference was noted with PGE₂ treatment.

Figure 4-8. Trabecular Area (mm²). There was no significant difference with PGE₂ treatment in dwarf and Lewis rats.
Figure 4-9. Total BMC (mg/mm) in the femoral diaphysis. The mean values were significantly lower in the PGE₂-treated dwarf (P=0.0002) and Lewis rats (P=0.0047), when compared to their respective vehicle-treated controls.

Figure 4-10. Total BMD for (mg/cm³). Dwarf rats treated with PGE₂ showed a significantly lower value than vehicle-treated controls (P<0.0001). No difference was observed in Lewis rats with PGE₂ treatment.
Figure 4-11. Cortical BMC (mg/mm). A significantly lower mean value was observed in the PGE$_2$ treated dwarf rats (P=0.0003), but not in PGE$_2$ treated Lewis rats (P=0.0910).

Figure 4-12. Cortical BMD (mg/cm$^3$). PGE$_2$-treated rats showed significantly lower mean values in both groups (P<0.0001).
Figure 4-13. Cortical Area (mm$^2$). Dwarf rats treated with PGE$_2$ had a significantly lower mean value ($P=0.0496$), but there was no difference with PGE$_2$ treatment in Lewis rats.

Figure 4-14. Cortical Thickness (mm). There was no significant difference with PGE$_2$ treatment in dwarf and Lewis rats.
Figure 4-15. Periosteal Circumference (mm). PGE$_2$-treated Lewis rats had a significantly lower mean value (P=0.0038), but there was no difference with PGE$_2$ treatment in dwarf rats (P=0.0826).

Figure 4-16. Endosteal Circumference (mm). A significant lower mean value was observed in PGE$_2$-treated Lewis rats (P=0.0002), but not in PGE$_2$-treated dwarf rats.
Figure 4-17. Vertebral Cancellous Bone Volume (%). There was no significant difference in PGE$_2$ treated dwarf and Lewis rats.

Figure 4-18. Vertebral Trabecular Number (#/mm). A significant increase was observed in Lewis (P=0.0004) and dwarf rats (P=0.0021) treated with PGE$_2$.
Figure 4-19. Vertebral Trabecular Width (μm). There was no significant difference in PGE$_2$-treated dwarf and Lewis rats.

Figure 4-20. Vertebral Trabecular Separation (μm). Significantly lower mean values were observed in PGE$_2$-treated dwarf (P=0.0041) and Lewis rats (P=0.0417).
Figure 4-21. Vertebral Osteoid Surface (%). No significant differences were observed with PGE₂ treatment in dwarf and Lewis rats.

Figure 4-22. Vertebral Osteoblast Surface (%). There were no significant differences in PGE₂-treated dwarf and Lewis rats.
Figure 4-23. Vertebral Osteoclast Surface (%). There were no significant differences with PGE$_2$ treatment in dwarf and Lewis rats.

Figure 4-24. Vertebral Mineralizing Surface (%). Only PGE$_2$-treated Lewis rats showed a significantly increase ($P=0.0068$) compared to their vehicle-treated controls.
Figure 4-25. Vertebral Bone Formation Rate ($10^{-2}\mu m^3/\mu m^2/d$). There were no significant differences with PGE$_2$ treatment in dwarf and Lewis rats.

Figure 4-26. Vertebral Cancellous Mineral Apposition Rate ($\mu m/d$). Only PGE$_2$-treated dwarf rats showed a significant decrease ($P=0.0030$) compared to their vehicle-treated controls.
Figure 4-27. Longitudinal Bone Growth (µm/d). Only PGE$_2$-treated dwarf rats showed a significant increase (P<0.0001) compared to their vehicle-treated controls.

Figure 4-28. Proximal tibial metaphyses from vehicle-treated (A) and PGE$_2$-treated dwarf (B) rats. Note the increased number of black-stained trabecular bone spicules and the wider growth plate in the PGE$_2$-treated dwarf rat (Von Kossa/tetrachrome stain, X20). Photos courtesy of Dr. Thomas J.Wronski.
Figure 4-29. Tibial Cancellous Bone Volume (%). PGE$_2$ induced a significant increase only in dwarf rats compared to their controls (P=0.0168)

Figure 4-30. Tibial Trabecular Number (#/mm). PGE$_2$-treated dwarf rats exhibited a significant increase compared to their controls (P=0.0056), but not PGE$_2$-treated Lewis rats
Figure 4-31. Tibial Trabecular Width (μm). PGE₂-treated Lewis rats showed a significantly lower value compared to their controls (P=0.0227), whereas there was no difference in PGE₂-treated dwarf rats.

Figure 4-32. Tibial Trabecular Separation (μm). There were no significant differences with PGE₂ treatment in dwarf and Lewis rats.
Figure 4-33. Total Cortical Bone Tissue Area (mm$^2$). There were no significant differences with PGE$_2$ treatment in dwarf and Lewis rats.

Figure 4-34. Cortical Area (mm$^2$). There was no significant difference in dwarf rats treated with PGE$_2$, but PGE$_2$-treated Lewis rats showed a significant decrease (P=0.0256).
Figure 4-35. Marrow Area (mm²). PGE₂-treated dwarf rats showed a significantly increased mean value compared to their vehicle-treated controls (P=0.0111), whereas PGE₂-treated Lewis rats showed no difference.

Figure 4-36. Periosteal Perimeter (mm). There were no significant differences with PGE₂ treatment in dwarf and Lewis rats.
Figure 4-37. Endocortical Perimeter (mm). PGE$_2$-treated dwarf rats showed a significantly increased value compared to their vehicle-treated controls (P=0.0273), whereas PGE$_2$-treated Lewis rats showed no difference.

Figure 4-38. Cortical Width (μm). PGE$_2$-treated Lewis rats showed a significantly decreased mean value compared to their vehicle-treated controls (P=0.0049), whereas PGE$_2$-treated dwarf rats showed no difference.
Figure 4-39. Periosteal Mineralizing Surface (%). There were no significant differences with PGE$_2$ treatment in dwarf and Lewis rats.

Figure 4-40. Periosteal Mineral Apposition Rate (um/d). PGE$_2$ treatment significantly increased the mean value in dwarf rats (P<0.0001), but not in Lewis rats.
Figure 4-41. Periosteal Bone Formation Rate ($10^{-2}$um$^3$/um$^2$/d). No significant differences were observed in dwarf and Lewis rats with PGE$_2$ treatment.

Figure 4-42. Endocortical Mineralizing Surface (%). PGE$_2$ treatment did not induce a significant effect in dwarf and Lewis rats.
Figure 4-43. Endocortical Mineral Apposition Rate (um/d). There was a significant increase in PGE$_2$-treated dwarf rats compared to their vehicle-treated controls (P<0.0001), but not in PGE$_2$-treated Lewis rats.

Figure 4-44. Endocortical Bone Formation Rate (10$^{-2}$um$^3$/um$^2$/d). PGE$_2$-treated dwarf rats exhibited a significant increase (P=0.0033), but not PGE$_2$-treated Lewis rats.
Figure 4-45. Biomechanical Load (N). PGE$_2$-treated dwarf and Lewis rats did not show significant differences compared to their vehicle-treated controls.

Figure 4-46. Biomechanical stress (N*mm$^2$). There was a significant increase in PGE$_2$-treated Lewis rats (P<0.0005), but not in PGE$_2$-treated dwarf rats.
Figure 4-47. Biomechanical Stiffness (N/mm). No significant differences were observed with PGE$_2$ treatment in both dwarf and Lewis rats.
CHAPTER 5
CHANGES IN GENE EXPRESSION RELATED TO BONE FORMATION AND BONE RESORPTION IN PTH- AND PGE₂-TREATED RATS.

Introduction

The polymerase chain reaction (PCR) has proven to be a reliable and powerful method to amplify a targeted DNA molecule and quantify tissue-specific gene expression. Basically, genes are transcribed by enzymes, such as RNA polymerases, thereby forming RNA. The characterization of RNA is almost always related to transcription (i.e., gene expression). The sequence of the RNA molecules correlates with the DNA from which they are derived (Livak and Schmittgen, 2001, Farrell Jr, 2005). Ultimately, all cell and tissue functions are governed by gene expression; in the skeleton, bone formation and resorption are under strict control of gene activation and suppression in response to physiological stimuli, hormones, and growth factors, including IGF-I and PTH.

Insulin Like Growth Factor I (IGF-I, IGF1, Somatomedin C)

According to the Gene-NICBI (National Center for Biotechnology Information) database, the IGF1 gene, chromosome location 12q23.2 (Homo sapiens), encodes the protein IGF-I, similar in structure to insulin, and is involved in mediating growth and development. In the skeleton, systemic IGF-I, regulated by growth hormone (GH), has an important role in linear growth and peak bone acquisition, and local IGF-I is involved in bone turnover. The literature is rich in studies demonstrating the effects of global, partial or tissue-specific deletion of the IGF-I gene (Powell-Braxton et al., 1993, Yakar et al., 1999, Rosen et al., 2004, He et al., 2006) on skeletal development and bone metabolism. The role of IGF-I as potential mediator for the bone anabolic effects of PTH has also been scrutinized in several in vivo and in vitro studies, since IGF-I gene
expression was found to be upregulated in bone cell cultures and in bone tissue from PTH-treated rats (Linkhart et al., 1989, Dempster et al., 1993, Gunness, 1995, Bikle et al., 2002). Several other studies showed that PGE$_2$ upregulates gene expression for IGF-I in bone cells, increasing IGF-I transcripts by 2.2 fold (McCarthy et al., 1991), and that IGF-I mRNA expression was correlated with osteogenesis (Harada et al., 1995).

However, the molecular and cellular mechanisms underlying the skeletal effects of PTH and PGE$_2$, and the role IGF-I as a potential mediator, are not completely clarified. To better understand the remarkable differences between the above mentioned studies and our previous findings with PTH and PGE$_2$ treatment in IGF-I deficient dwarf rats, we evaluated the changes in the abundance of IGF-I and six other genes involved in bone metabolism.

**Collagen Type I**

The organic mass of bone matrix is composed of about 90% collagen type I, the most abundant protein in vertebrates, providing the structural framework of the skeleton. It is also responsible for bone shape and its biomechanical properties such as resistance to pressure, torsion, and tension (Mark, 2006). In humans, collagen type I is composed by two $\alpha_1$chains and one $\alpha_2$ chain, that are coded by different genes Col1a1 and Col1a2, which are very similar in structure. Mutation in either of these genes causes a genetic disease called osteogenesis imperfecta, or “brittle bone disease”, characterized by a decrease in bone mass, enhanced fragility and multiple fractures. Type I collagen synthesis can be modified by hormones, cytokines, vitamins, and growth factors, including IGF-I (Bou-Gharios and Crombrugghe, 2008). Type I collagen is synthesized by osteoblasts, and its gene expression is therefore indicative of bone formation.
Osteocalcin

Osteocalcin is a bone specific protein, secreted by osteoblasts, that participates in bone mineralization and calcium homeostasis. It is also known as bone gamma-carboxyglutamic acid-containing protein (BGLAP), encoded by the BGLAP or OC gene, and accounts for up to 20% of noncollagenous protein in bone, with an affinity for bone mineral. However, when the affinity for hydroxyapatite is reduced in its uncarboxylated form, the osteocalcin molecule can enter the systemic circulation more easily and function as a hormone, regulating glucose metabolism, energy expenditure and fat mass (Lee et al., 2007, Clemens and Karsenty, 2011). Recent studies, in vitro and in vivo, revealed that osteocalcin increases the number of pancreatic β-cells, insulin secretion and sensitivity, and release of adiponectin by fat cells (Lee et al., 2007, Ferron et al., 2008, Yoshikawa et al., 2011).

The osteocalcin gene is not expressed in nonosseus cells, or even in osteoprogenitor cells. Its transcription is controlled by the runt-related transcription factor 2 (Runx2), following osteoblast differentiation (Stein et al., 2008). Due to its specificity, osteocalcin is commonly used as a biomarker for bone formation.

Osterix

Osterix (Osx) is a transcriptional factor required for osteoblast differentiation and bone formation. When first identified by Nakashima in 2002, as a novel zinc finger-containing transcription factor, osterix was thought to be specifically expressed in developing bone cells, and required for osteoblast differentiation during embryonic development. No bone formation occurred when Osx expression was deleted in mice, which died at birth. Mesenchymal cells did not deposit bone matrix and cells in the periosteum did not differentiate into osteoblasts (Nakashima et al., 2002). However,
Zhou (2010) demonstrated that osterix is also required for bone growth and homeostasis in the postnatal period, by deleting Osx in mice at several different point times postnatally. It was also noticed that the inactivation of Osx caused severe disruption in the morphology, maturation and function of osteocytes, as well as accumulation of unresorbed calcified cartilage below the growth plate (Zhou et al., 2010).

**RANKL**

The receptor activator of nuclear factor-kappa β ligand (RANKL) is also known as tumor necrosis factor-related activation-induced cytokine (TRANCE), osteoprotegerin ligand (OPGL), and osteoclast differentiation factor (ODF). RANKL is a transmembrane ligand expressed in osteoblasts and bone marrow cells. It binds to RANK (receptor activator of nuclear factor-kappa β), which is expressed in osteoclast progenitor cells to induce osteoclastogenesis, in the concomitant presence of M-CSF (macrophage colony stimulating factor) (Asagiri and Takayanagi, 2007). In addition to osteoclast differentiation, RANKL is also essential to induce osteoclast activation and, consequently, bone resorption. RANK is present on the cell surface of mature osteoclasts, and in response to activation by its ligand, the osteoclast goes through structural changes that initiate bone resorption. Changes occurring in the osteoclast’s actin cytoskeleton are followed by formation of a sealed compartment adjacent to the bone surface, with release of lytic enzymes and consequent erosion of the underlying bone (Boyle et al., 2003). Therefore, RANKL is the most important cytokine involved in osteoclast differentiation and activation, being essential for bone resorption. RANKL is encode by the *Tnfs11* gene and RANK by the *Tnfs11a* gene. Mice with deletion of
either of these genes exhibit severe osteopetrosis and defective tooth eruption due to a complete lack of osteoclasts. In osteopetrosis, the defect in bone turnover due to the lack of osteoclastogenesis results in skeletal fragility despite an increase in bone mass, growth impairment, and hematopoietic insufficiency (Nakashima et al., 2012). The genetic ablation of RANK also leads to defects in immune system cells (B- and T-cells), demonstrating an additional role for RANKL in lymph node formation (Dougall et al., 1999).

**Osteoprotegerin (OPG)**

Osteoprotegerin was first described in 1999 as a novel glycoprotein, and a member of the TNF (tumor necrosis factor) superfamily that regulates bone resorption (Simonet et al., 1997). OPG participates in bone metabolism by competing with RANKL for binding to RANK, as it shows a strong homology to RANK receptors expressed in several tissues, including osteoblast lineage cells in bone. OPG acts as a decoy receptor for RANKL and therefore inhibits RANKL-mediated osteoclastogenesis and the survival of pre-existing osteoclasts (Lian and Stein, 2006). The balance between RANKL and OPG determines osteoclast formation and bone resorption activity. OPG is encoded by *Tnfs11b* gene and its deletion in OPG-/- mice results in an excess of RANKL activity, with spontaneous fractures and vertebral deformities, although skeletal growth is not impaired (Feige, 2001).

**Sclerostin**

Sclerostin is a glycoprotein encoded by the SOST gene and a potent negative regulator of bone formation (Keller and Kneissel, 2005). Sclerosteosis is an autosomal recessive condition characterized by progressive and generalized osteosclerosis, with enlargement of the jaw, thickening of the skull, sometimes causing cranial nerve
entrapment and eventually potential loss of smell and hearing, gigantism and syndactyly (Whyte, 2006, Canalis et al., 2007, Bezooijen et al., 2008). Although it is a rare bone disorder, sclerosteosis generated considerable interest due to the resulting phenotype that indicated an imbalance in bone metabolism in favor of bone formation. The bone formed was of overall good quality, with increased bone volume and bone mineral density without occurrence of pathological fractures (Bezooijen et al., 2008). In 2001, sclerosteosis was related to the deactivating mutation in the gene encoding sclerostin (SOST), and five mutations of the SOST gene have been identified to date (Whyte, 2006). Sclerostin is a member of the DAN (differential screening-selected gene aberrant neuroblastoma) that shares the ability to antagonize bone morphogenetic proteins (BMPs). Sclerostin is also reported to antagonize Wnt signaling (mammalian homologue of drosophila gene wingless, that induces differentiation of bone-forming cells), binding to the receptors LRP (lipoprotein receptor-related protein) 5 and 6 (Canalis et al., 2007, Bezooijen et al., 2008). The inhibition of sclerostin production and/or activity by monoclonal antibodies against sclerostin, preventing its binding to Wnt receptors, can enhance Wnt signaling and increase bone mass in rodents and nonhuman primates (Warmington et al., 2005). Sclerostin antibody treatment has been evaluated in animal models, and it was reported to increase bone formation, bone mass and strength in a rat model for postmenopausal osteoporosis (Li et al., 2009), and increase bone healing in rats with metaphyseal fractures (Agholme et al., 2011). Its clinical applicability must be confirmed to avoid unwanted nonskeletal effects. Sclerostin is synthesized and secreted primarily by osteocytes. SOST is considered a target gene for the bone anabolic actions of PTH. Its osteoblastogenic effects appear to
result from the inhibitory effects of PTH on sclerostin synthesis by osteocytes (Keller and Kneissel, 2005, Bellido, 2006).

Materials and Methods

Animal Models and Experimental Design

Dwarf rats express significantly decreased IGF-I serum levels compared to their background strain, Lewis rats, representing a major reduction of 60% in circulating IGF-I. Therefore, the dwarf rat is a reliable model to study the physiological changes seen in IGF-I deficiency. In addition, GH/IGF-I deficiency in the dwarf rat has profound negative effects on bone growth, accumulation of bone mass, and osteoblast activity in both cortical and cancellous bone. For this reason, it is an adequate animal model to study the osteopenic effects caused by IGF-I deficiency. Since IGF-I may mediate the skeletal effects of bone anabolic agents, the dwarf rat is also a promising animal model for studies of these interactions.

Female Lewis (background strain) and dwarf rats were obtained from Harlan Laboratories (UK). These animals were distributed in 6 experimental groups, as follows:

1. Female VEH-treated Lewis rats (N=13).
2. Female VEH-treated Dwarf rats (N=13).
3. Female PTH-treated Lewis rats (N=7).
4. Female PTH-treated Dwarf rats (N=10).
5. Female PGE2-treated Lewis rats (N=7).
6. Female PGE2-treated Dwarf rats (N=7).

They were housed two per cage, with relative humidity, air quality, illumination (12h light/12h dark cycles), and temperature controlled according to the criteria established by the Institute for Laboratory Animal Research and the National Research
Council (2011). The protocol for use of rats in this research project was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Florida.

At 9 weeks of age, the rats were injected subcutaneously, daily for 2 weeks with vehicle, hPTH 1-34 at a dose of 50 μg/kg body weight, or PGE₂ at a dose of 3 mg/kg body weight, according to their experimental groups. At the end of the treatment period, all rats were euthanized by exsanguination under ketamine/xylazine anesthesia.

**RNA Extraction and cDNA Synthesis**

RNA integrity is critical for all gene expression analyses, and to reliably use real-time RT-PCR, high quality and undegraded RNA is required.

**Bone tissue**

Distal femora were selected in order to obtain greater RNA yields due to the size of the bones and the higher proportion of trabecular to cortical bone (Li et al., 2008). The bone samples were collected at the time of euthanasia, dissected free of surrounding soft tissue, snap frozen in liquid nitrogen and stored at -80°C until used for RNA extraction, with Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions adapted for bone tissue. Bone is a mineralized tissue, containing an abundant matrix rich in degradative enzymes, which makes the isolation of non-degraded RNA, free from inhibitors, a major challenge in the analysis of gene expression in the skeleton (Ireland, 2003). For these reasons, before the Trizol extraction, bone samples were pulverized using a 6750 Freezer Mill and Auto Extractor 6814 (Spex CertiPrep, Metuchen, NJ) (Figure 5-1) to guarantee adequate cellular disruption, as RNA is isolated from the nucleus or cytoplasm by direct cell lysis (Farrell Jr, 2005). This resulted in good quality pellets, of large size and easily visible, after the extraction protocol (Figure 5-2).
Following RNA extraction, the concentrations were determined with a Nanodrop spectrophotometer (ND-1000, ThermoFisher, Wilmington DE), and based on these values, all samples were treated with an RNase-free DNase kit (Qiagen RNeasy Plus), to remove any residual genomic DNA. The concentrations were re-evaluated for quantity control, then sent to the Interdisciplinary Center for Biotechnology Research at the University of Florida (ICBR) for quality control. The integrity of the RNA was measured by capillary electrophoresis using an Agilent Bioanalyzer, 2100 model, that provides an improved method to visualize nucleic acids, as software generates gel-like images, an electropherogram, and displays sample concentration and ribosomal ratio, as shown in one of our reports in Figure 5-3. If the obtained RNA integrity number (RIN) did not reveal good RNA quality, the sample was excluded from further analysis. The assessment of RNA integrity is a critical step in obtaining reliable gene expression data. The RNA samples were converted to cDNA with a High Capacity cDNA Archive kit, following the manufacturer’s protocol (Applied Biosystems, Foster City, CA). Once synthesized, the cDNA was stored at –20°C until quantitative real time PCR (qRT PCR) was performed.

**Hepatic tissue**

Liver samples were homogenized with Trizol (Invitrogen, Carlsbad, CA) with a TissueLyser LT (Qiagen) machine, that speeds the extraction process for soft tissue samples. RNA extraction was then performed following the same protocol as for bone samples, with the same quantity and quality control, and converted to cDNA.

**Quantitative Real Time-PCR**

Primers and probes for each gene were designed from the corresponding murine (*Rattus norvegicus*) mRNA in the NCBI Reference Sequence (National Center for
Biotechnology Information Gene Database), with Primer Express software (Applied Biosystems), as seen in Figures 5-4 to 5-10. The optimization and efficiency of the primers and probes were performed for all seven genes before real-time PCR reactions. The abundance of 18S rRNA (Applied Biosystems), used as endogenous control, was also measured for each sample.

The relative expression of all seven genes, IGF-I, Collagen Type I, Osteocalcin, Osterix, RANKL, OPG, and Sclerostin were determined by qRT-PCR using FAM Taqman probes and primers, and Taqman RT-PCR master mix (Applied Biosystems, Foster City, CA). All samples were run in triplicate for each gene and for 18S rRNA. Each plate was set up to include sample unknowns from all experimental groups (vehicle, PTH- and -PGE$_2$-treated rats) for a single gene, plus no-template controls from each primer/probe pairs. For liver samples, only the IGF-I gene was evaluated, in order to compare the abundance of IGF-I synthesized in the liver to the local IGF-I produced in bone tissue, using the same endogenous control (18S rRNA).

**Calculations and Statistical Analysis**

In each PCR assay, the cycle threshold (Ct) value was obtained in triplicate for each sample, for each gene, and in all groups. Relative mRNA expression of each gene was calculated by determining the change in threshold cycle (ΔCt) between the mean Ct for each gene and the mean Ct for 18S rRNA mRNA from the same sample. Values for ΔCt were statistically analyzed by ANOVA (analysis of variance), followed by a multiple comparison test (Scheffe post hoc analysis, StatView - SAS Software Institute Inc.) to compare the effects of PTH and PGE$_2$ on expression of each gene. Differences were considered significant at P<0.05. Data are depicted in graphs as the mean fold change in mRNA relative to the control group (vehicle treated-rats). The fold change
was calculated using the $2^{\Delta \Delta CT}$ method, as described by Livak (2001), where $\Delta \Delta Ct$ is the difference between $\Delta Ct$ in each sample and the mean $\Delta Ct$ in the control group (Livak and Schmittgen, 2001).

**Results**

**IGF-I**

**Bone**

There was no significant difference in IGF-I gene expression in local bone tissue between dwarf and Lewis rats ($P=0.9956$). The expression of IGF-I was significantly decreased by PGE2 treatment in dwarf ($P<0.0001$) and Lewis rats ($P<0.0001$). PTH treatment induced a slight decrease in the expression of IGF-I in dwarf rats ($P<0.0506$), but no significant difference was observed in Lewis rats ($P=0.0946$), as seen in Figures 5-11 and 5-12.

**Liver**

IGF-I expression in the liver was not significantly different between vehicle-treated dwarf and Lewis rats ($P=0.9994$), and between PGE2-treated ($P=0.1646$) and PTH-treated dwarf rats ($P=0.9912$) compared to their vehicle-treated controls. PGE2 treatment induced a significant increase in liver IGF-I expression in Lewis rats ($P<0.0115$), and there was a strong trend for an increase in PTH-treated Lewis rats ($P<0.0562$), as seen in Figure 5-13 and 5-14.

**Collagen Type I**

There was no significant difference in collagen type I gene expression when comparing vehicle-treated dwarf and Lewis rats ($P=0.9966$). Only PGE2 treatment induced a significant decrease in the expression of collagen type I in both dwarf and
Lewis rats. There were no significant differences caused by PTH treatment in dwarf (P=0.9628) and Lewis rats (P=0.9811), as seen in Figures 5-15 and 5-16.

**Osteocalcin**

The expression of osteocalcin was affected by PGE\(_2\) treatment, showing a significant decrease in both dwarf (P<0.0001) and Lewis rats (P<0.0004), but PTH treatment induced a significant decrease only in dwarf rats (P<0.0058), with no effect in Lewis rats (P=0.9998), as seen in Figures 5-17 and 5-18.

**Osterix**

Osterix gene expression was significantly decreased in PGE\(_2\)-treated dwarf (P<0.0001) and Lewis rats (P<0.011), whereas PTH treatment did not induce any significant changes in dwarf (P=0.999) or Lewis rats (P=0.7968), as seen in Figures 5-19 and 5-20.

**RANKL**

There was a statistically significant decrease in RANKL gene expression in PGE\(_2\)-treated dwarf (P<0.0001), PGE\(_2\)-treated Lewis (P<0.0001), and PTH-treated Lewis rats (P<0.005), but no effect in PTH-treated dwarf rats (P=0.1235), as seen in Figures 5-21 and 5-22.

**OPG**

There was a statistically significant decrease in OPG gene expression in PGE\(_2\)-treated dwarf (P<0.0001) and Lewis rats (P<0.0001), but no significant changes were induced by PTH treatment in dwarf (P=0.2787) and Lewis rats (P=0.0603), as seen in Figures 5-23 and 5-24.
**Sclerostin**

Gene expression for sclerostin was affected by both treatments in dwarf rats, with statistically significant decreases for PGE$_2$ (P<0.0004) and PTH (P<0.001). In Lewis rats, gene expression for sclerostin was significantly decreased by PGE$_2$ treatment, but no significant change was observed with PTH treatment (P=0.2032), as seen in Figure 5-25 and 5-26.

**Discussion**

Surprisingly, our findings did not reveal a significant difference in the IGF-I gene expression in the liver, nor in the local bone tissue of vehicle-treated dwarf and Lewis rats. These results contrast strongly with the decreased serum levels observed in dwarf rats, regardless if treated with vehicle, PTH or PGE$_2$. We speculate that if RNA transcription is not affected, as indicated by our findings, the production of IGF-I can be altered at the translational level, with a decrease in protein synthesis, and consequently in serum levels. Similarly, in other studies, protein levels did not reflect changes in RNA abundance (Wood and Giroux, 2003). Flyvbjerg et al. also used dwarf rats to study the effects of isolated growth hormone and IGF-I deficiency on diabetic renal hypertrophy, and observed that despite transient kidney IGF-I accumulation, no increase was found in kidney mRNA during the first 4 days of the onset of diabetes, indicating that local IGF-I synthesis at the transcriptional level was unchanged (Flyvbjerg et al., 1992). In another study with GH deficient (dw/dw) rats, hIGF-I treatment was associated with increased longitudinal growth, weight gain, and increased plasma IGF-I levels, but not in hepatic IGF-I mRNA expression, when compared to saline control treatment. Insulin treatment, however, increased the hepatic IGF-I mRNA expression, with a reduction in plasma IGF-I levels (Butler et al., 1996). However, male Wistar rats
hypophysectomized on postnatal day 26, showed markedly reduced serum GH and IGF-I levels compared to intact controls, as well as lower IGF-I mRNA levels in the liver (Domene et al., 1993). IGFBP-3 mRNA levels were also reduced in hypophysectomized animals. Considering that hepatic IGFBP-3 and ALS synthesis are stimulated by GH (Molina, 2006a), and that they form the ternary complex that represents 80% of the circulating IGF-I, we hypothesize that if the IGFBP-3 production is decreased at any level, it could also affect the bioavailability of IGF-I in the circulation and tissues as well. IGFBPs and ALS were not evaluated in our experiments. It is also relevant that the dwarf rats not only presented lower levels of serum IGF-I, but marked phenotypic changes in bone structure and metabolism were detected by histomorphometric and pQCT analyses in our previous experiments.

RANKL was significantly decreased in PGE$_2$-treated dwarf and Lewis rats, or showed a trend for a decrease in both groups of PTH-treated rats, while OPG gene expression was decreased by only PGE$_2$ treatment in dwarf and Lewis rats. Although unclear, since PTH is considered to participate in the regulation of RANKL and OPG (Huang et al., 2004), these findings did not lead us to emphasize the involvement of these two main regulators of bone resorption on the observed bone anabolic effects, mainly with PTH treatment. Type I collagen, osteocalcin and osterix gene expression were not induced by PTH and PGE$_2$ treatments in a manner consistent with the observed increase in bone formation. However, the decreased expression of the SOST gene in dwarf rats treated with PTH and PGE$_2$ is consistent with studies that relate this gene to the anabolic effects of PTH, through a direct inhibitory effect on osteocytic synthesis of sclerostin and its antagonistic action on the Wnt signaling pathway (Bellido,
Keller (2005) demonstrated that PTH strongly suppresses SOST expression in vivo and in vitro, and considered SOST a direct target gene for PTH, regulated at the transcription level (Keller and Kneissel, 2005). Mice with overexpression of the SOST gene were also found to have a blunted response to PTH-induced bone gain (Kramer et al., 2010).

Interestingly, the dwarf rats, deficient in IGF-I, showed a significant decrease in SOST gene expression with PTH treatment, while Lewis rats, with normal IGF-I levels, did not. This finding contrasts with several studies, in vitro and in vivo, that postulate a role for IGF-I as an essential mediator for the stimulatory effects of PTH on bone formation (Canalis et al., 1989, Dempster et al., 1993, Gunness, 1995, Bikle et al., 2002).

**Conclusions**

The major findings were:

1. Gene expression for IGF-I in bone tissue did not show a significant difference between IGF-I deficient dwarf rats and their background strain, Lewis rats.

2. Although dwarf rats show lower levels of serum IGF-I, gene expression for IGF-I in the liver was not significantly different compared to Lewis rats.

3. In dwarf rats, PTH treatment did not change the abundance of IGF-I mRNA in bone or liver, nor the expression of genes related to bone formation: collagen type I, osteocalcin, and osterix. Genes related to bone resorption, RANKL and OPG, also did not show any significant differences in PTH-treated dwarf rats.

4. PGE2 treatment induced significant decreases in the gene expression for IGF-I in bone (liver IGF-I decreased only in Lewis rats) and all the genes involved in bone formation (collagen type I, osteocalcin, and osterix) and bone resorption (RANKL and OPG).

5. SOST/sclerostin gene expression was downregulated in dwarf rats treated with PTH and PGE2, despite their low IGF-I serum levels.
Figure 5-1. Extractor (A) and freezer mill (B) used to pulverize bone tissue. Photos courtesy of Dr. Ana Cristina F. Bassit

Figure 5-2. RNA pellets obtained from bone samples. Photo courtesy of Dr. Ana Cristina F. Bassit
Figure 5-3. Bioanalyzer results indicating high RNA quality and integrity
Figure 5-4. IGF-I primers and probe sequences obtained with Primer Express 3.0
Figure 5-5. Collagen Type I primers and probe sequences obtained with Primer Express 3.0

![Image of a software interface showing sequence and location information for primers and probes.](image-url)
Figure 5-6. Osteocalcin primers and probe sequences obtained with Primer Express 3.0.
Figure 5-7. Osterix primers and probe sequences obtained with Primer Express 3.0
Figure 5-8. RANKL primers and probe sequences obtained with Primer Express 3.0
Figure 5-9. Osteoprotegerin primers and probe sequences obtained with Primer Express 3.0
Figure 5-10. Sclerostin primers and probe sequences obtained with Primer Express 3.0
Figure 5-11. Fold change in bone IGF-I expression in dwarf rats (RQ=relative quantity)

Figure 5-12. Fold change in bone IGF-I expression in Lewis rats
Figure 5-13. Fold change in liver IGF-I expression in dwarf rats

Figure 5-14. Fold change in liver IGF-I expression in Lewis rats
Figure 5-15. Fold change in collagen type I gene expression in dwarf rats

Figure 5-16. Fold change in collagen type I gene expression in Lewis rats
**Figure 5-17.** Fold change in osteocalcin gene expression in dwarf rats

**Figure 5-18.** Fold change in osteocalcin gene expression in Lewis rats
Figure 5-19. Fold change in osterix gene expression in dwarf rats

Figure 5-20. Fold change in osterix gene expression in Lewis rats
Figure 5-21. Fold change in RANKL gene expression in dwarf rats

Figure 5-22. Fold change in RANKL gene expression in Lewis rats
Figure 5-23. Fold change in OPG gene expression in dwarf rats

Figure 5-24. Fold change in OPG gene expression in Lewis rats
Figure 5-25. Fold change in sclerostin gene expression in dwarf rats

Figure 5-26. Fold change in sclerostin gene expression in Lewis rats
Summary of Experimental Findings

Study 1 Summary

In study 1, we explored the potential use of the dwarf rat as an animal model to study the effects of deficiency in the GH/IGF-I axis. The overall growth and development of the dwarf rat were compared to those observed in their background strain, the Lewis rat. Body weight and femur length were evaluated throughout the study. IGF-I serum levels were measured and we systematically analyzed changes in the skeleton and bone tissue by histomorphometry, pQCT and biomechanical testing. Our results showed that body weight and femur length were significantly decreased in the dwarf rat, compared to the Lewis rat. Most importantly, the dwarf rat expressed significantly decreased IGF-I serum levels compared to its background strain, Lewis rats, representing a major reduction of 60% in circulating IGF-I.

In addition, the dwarf rat expressed an evident osteopenic phenotype, with profound negative effects on bone growth, accumulation of bone mass, and osteoblast activity in both cortical and cancellous bone. Consequently, decreased bone strength was also observed, with significantly lower load to failure in dwarf rats. For these reasons, we concluded that the dwarf rat is an adequate animal model to study physiological changes seen in IGF-I deficiency and the related osteopenic effects on bone structure and metabolism. Since IGF-I may mediate the skeletal effects of bone anabolic agents; the dwarf rat is also a promising animal model for studies of these interactions.
Study 2 Summary

Based on the results of study 1, the experiments in study 2 were designed using the same methods to test the bone anabolic effects of intermittent PTH treatment in IGF-I deficient dwarf rats. We observed that PTH did not affect body weight or serum IGF-I levels in either Lewis or dwarf rats. However, PTH stimulated both cancellous and cortical bone formation, and induced highly significant anabolic effects in vertebral and tibial cancellous bone in dwarf rats, despite low circulating levels of IGF-I.

Study 3 Summary

Similarly to study 2, the experiments in study 3 were also based on the methods and results obtained in study 1, but designed to evaluate the effects of PGE2 on bone mass and formation. PGE2 treatment did not increase body weight or serum IGF-I levels in either Lewis or dwarf rats, but our results indicated that PGE2 treatment increased bone mass in dwarf rats, mainly in cancellous bone, despite lower IGF-I serum levels.

Study 4 Summary

In study 4, PCR assays were performed to evaluate changes in the expression of genes involved in bone formation and bone resorption in response to PTH and PGE2 treatments in dwarf and Lewis rats. Liver IGF-I gene expression was also evaluated, as an attempt to differentiate the expression of local from systemic IGF-I. The results of this study demonstrated that, although dwarf rats showed lower levels of serum IGF-I, gene expression of IGF-I in the liver was not significantly different compared to Lewis rats. Similarly, gene expression of IGF-I in bone tissue did not show a significant difference between IGF-I deficient rats and their background strain, Lewis rats.
The results obtained in study 4 also demonstrated that PTH treatment did not affect the abundance of IGF-I in bone or liver, nor the genes related to bone formation: collagen type I, osteocalcin, and osterix in dwarf rats. Genes related to bone resorption, RANKL and OPG, were not affected by PTH treatment either. PGE$_2$ treatment induced a significant decrease in gene expression of IGF-I in bone (liver IGF-I decreased only in Lewis rats) and all the genes involved in bone formation (collagen type I, osteocalcin, and osterix) and bone resorption (RANKL and OPG). SOST/sclerostin gene expression was downregulated in dwarf rats treated with PTH and PGE$_2$, despite their low IGF-I serum levels.

**Discussion**

In study 1, we explored the potential use of the dwarf rat as an animal model to study the effects of deficiency in the GH/IGF-I axis. Several animal models have been developed for this purpose, since 1959, and even earlier, when ordinary laboratory rats, by that time merely described as albino rats, were hypophysectomized and treated with growth hormone. Korner (1959) observed that hypophysectomized female albino rats, not only showed a decrease in their body weight and organ size, but also a diminished incorporation of amino acids into proteins in hepatic cells *in vitro*, strongly suggesting that protein biosynthesis *in vivo* was also decreased. It was also demonstrated that these effects could be suppressed with growth hormone treatment (Korner, 1959). Since these early studies, many different strains of genetically modified animal models were created to investigate IGF-I deficiency. These mutant animals have been unquestionably useful in studies for the understanding of cellular and biochemical pathways involved in the regulatory role of IGF-I, as an endocrine and paracrine hormone, in skeletal development and mineral acquisition. However, among the
recently compiled 55 different mouse models, only 26 of the created genetic mutations refer to a similar condition in the context of human IGF-I deficiencies (Yakar et al., 2010).

Reproducing the characteristics of a disease in a reliable manner is a major requirement when selecting the most adequate animal model for research. One of our main goals was to evaluate the effects of IGF-I deficiency on the skeleton and bone metabolism, but in close similarity to the conditions presented in clinical cases. In this category we consider not only osteoporosis in postmenopausal women, but also other disorders that lead to decreased IGF-I levels and osteopenia, including the juvenile onset of osteoporosis, anorexia nervosa and hypothalamic amenorrhea, glucocorticoid-induced-osteoporosis, chemotherapy, and alcohol abuse. This was one of the reasons why knockout animals were not selected for our studies, not to mention the poor health status of these animals.

Since we also wanted to evaluate how GH/IGF-I deficiency would affect bone growth, young rats were used in our studies, even though it was clear that their age could be considered a limitation to the applicability of our results to adult osteoporosis and other age-related conditions in which IGF-I levels are decreased. When evaluating the rat as an animal model for osteoporosis studies in the past, many researchers considered that these rodents were not suitable due to predominant modeling activity (rather than remodeling) in cancellous bone and the lack of Haversian remodeling in cortical bone. In addition, osteoporosis in humans causes spontaneous and low-impact fractures, whereas the rat does not develop fragility fractures (Jee and Yao, 2001). However, bone remodeling is now known to occur in cancellous bone of adult rats
Furthermore, it has been recently considered that most of the protocols to induce osteopenia and osteoporosis, by hormonal (surgical or pharmaceutical) and dietary interventions, as well as by immobilization, can be used in skeletally immature or mature rats, if low peak bone mass is achieved (Lelovas et al., 2008). The dwarf rats in study 1 presented evident osteopenic features, with negative effects on bone growth and accumulation of bone mass. When considering drug development and approval, guidelines for the FDA and the European Agency for the Evaluation of Medicinal Products (EMEA) require that new potential agents for osteoporosis therapy must be tested in two animal species: rodents (preferably rats) and a non-rodent, large animal model (Thompson et al., 1995, Avouac, 2003, Bagi et al., 2011).

Rats reach sexual and skeletal maturity when they are about 2.5 and 10 months old, respectively, although in some long bones, the epiphyseal plate remains open after 30 months (Jee and Yao, 2001, Lelovas et al., 2008). The female dwarf and Lewis rats chosen for our studies were five weeks old at the beginning of the experiments; PTH and PGE₂ treatments started when they reached 9 weeks of age, and they were euthanized at 11 weeks. Therefore, the results obtained can be extrapolated to juvenile patients affected by anorexia nervosa (AN), a condition characterized by severe weight loss and self-induced chronic starvation associated with decreased IGF-I serum levels, severe bone loss, and a two- to three-fold increase in fractures (Hofman et al., 2009, Lawson et al., 2010, Jacobson-Dickman and Misra, 2010, Misra and Klibanski, 2011). It occurs predominantly in women, affecting 0.2 to 1% of adolescent girls and 1 to 4% of college-age young women, although the incidence in men has increased in recent years (Jacobson-Dickman and Misra, 2010). In a study of AN prevalence, demographic data
of 286 female patients (range 15-54 years), the average age was 25 years, with 53 patients younger than 18 years (Hofman et al., 2009). AN presents critical negative effects on growth and maturation of the skeleton, and bone mass accrual during a period in life in which peak bone mass should be reached. These residual deficits in bone mass and accrual persist even after achieving weight gain and resuming menstrual function with treatment, representing a higher risk of developing osteoporosis later in adulthood (Jacobson-Dickman and Misra, 2010, Misra and Klibanski, 2011). Malnutrition, and estrogen and androgen deficiency are related to lower IGF-I levels and resistance to GH (Gianotti et al., 1998, Jacobson-Dickman and Misra, 2010). Systemic IGF-I levels are markedly decreased in patients with AN compared to normal subjects (Gianotti et al., 1998), as well as the serum levels of acid-labile subunit (ALS) (Fukuda et al., 1999) and IGFBP-3, whereas IGFBP-2 levels are increased (Hotta et al., 2000). Nutrition participates in the regulation of IGF-I and other anabolic hormones, and low IGF-I levels in anorectic patients are correlated with decreased BMD and with negative effects on bone architecture, represented by decreased bone volume, trabecular number, trabecular thickness and increased trabecular separation (Lawson et al., 2010).

When comparing the results obtained with PTH and PGE$_2$ treatments in studies 2 and 3, it becomes evident that the effects of PTH on bone mass and formation were superior to those of PGE$_2$, both in cancellous and cortical bone of dwarf and Lewis rats. In addition, we observed adverse side effects with PGE$_2$ treatment, such as acute abdominal pain, usually related to increased motility in the gastrointestinal and genitourinary tracts. In study 2, the surprisingly strong bone anabolic effects of PTH in IGF-I deficient dwarf rats introduced new questions about the bioavailability and
regulation of IGF-I, IGFBPs, and ALS; about their synthesis and action in the local tissues; about how they are influenced, by other hormones, such as PTH; and what mechanisms are involved. It is known that IGF-I knockout mice do not respond to PTH treatment (Miyakoshi et al., 2001, Bikle et al., 2002), but it is not understood if, when IGF-I levels are decreased, instead of abrogated, which levels would be considered as the minimum required to allow the bone anabolic action of PTH. Knockout mice were extensively used to demonstrate that not only IGF-I was required for the PTH anabolic actions on bone, but also its receptor IRS-1 (Wang et al., 2007), and the two other components of the circulating ternary IGF-I complex, ALS and IGFBP-3 (Yakar et al., 2006). However, the balance between the systemic and local actions of IGF-I is complex, and so is the regulation by six different binding proteins, and the interaction of IGF-I with other hormones, notably GH and PTH (Yakar et al., 2010, Bikle and Wang, 2011).

Still, we could refer to one study, by Miyakoshi (2001), who also tested whether low levels of systemic IGF-I would restore the actions of PTH on bone, comparing IGF-I knockout mice, IGF-I midi mice (an incomplete knockout for IGF-I), and wild-type controls. Total BMC and areal BMD, measured by DEXA, and total BMD measured by pQCT, were significantly increased by PTH treatment in IGF-I midi mice compared to the control group, whereas there was no significant effect of PTH treatment in IGF-I knockout mice. Serum IGF-I levels in IGF-I midi mice were considered 30% of normal, based on literature reference, but were not measured in this experiment, and no bone histologic analysis was performed (Miyakoshi et al., 2001).
Collagen type I gene expression was downregulated in both dwarf and Lewis rats treated with PGE$_2$, and we considered that it could reflect, in part, the heterogeneity of the results observed in pQCT and histomorphometric analyses. For example, PGE$_2$ treatment increased trabecular BMD and BMC only in dwarf rats, but cortical bone parameters were decreased. Cancellous bone volume was increased only in the proximal tibia of dwarf rats, but not in the lumbar vertebra, although there was an increase in the number of trabeculae. There are numerous growth factors, such as IGFs, BMPs, FGFs, steroid hormones and cytokines involved in the regulation of collagen synthesis and metabolism in bone (Mark, 2006).

Despite its bone anabolic effects in \textit{in vivo} studies, in many cell culture systems, PGE$_2$ has been shown to affect collagen by increasing its degradation and also to inhibit collagen synthesis, although the receptor and signaling mechanisms for the effects of PGE$_2$ are not fully explained (Raisz et al., 1993b, Fall et al., 1994). PGE$_2$ stimulates IGF-I synthesis in osteoblast cell cultures from rat bone (McCarthy et al., 1991), but inhibits alpha 1 procollagen gene transcription in osteoblastic cell cultures, in the presence or absence of IGF-I (Raisz et al., 1993b). Similarly, PGE$_2$ also inhibits type I collagen gene expression in fibroblasts at the transcriptional level (Goldring et al., 1996, Riquet et al., 2000), and delays chondrocyte maturation acting on BMP signaling in murine cell cultures (Clark et al., 2009). PGE$_2$ treatment also decreased osteocalcin gene expression in dwarf and Lewis rats. In cultures of a human osteoblast-like cell line MG-63 treated with bovine PTH (1-34) and PGE$_2$, a significant inhibition of osteocalcin secretion in response to the active form of vitamin D3 was observed (Lajeunesse et al., 1991).
PTH treatment did not induce any significant difference in the mRNA abundance of collagen type I in dwarf and Lewis rats, as would be expected, but *in vitro* results are conflicting. Kream (1980) observed that there was a 50% reduction in collagen synthesis and a 40% decrease in procollagen mRNA levels in fetal rat calvaria cell cultures, an effect detectable after 6h of PTH treatment (Kream et al., 1980), whereas Thiebaud (1994) found that PTH increased alpha 1collagen mRNA levels in osteoblastic cell cultures (Thiebaud et al., 1994).

Osteocalcin is the most abundant non-collagenous protein in bone tissue, synthesized by mature osteoblasts. PTH stimulates bone formation mainly by targeting cells of the osteoblast lineage. PTH increases osteoblast number and activity, decreases osteoblast apoptosis, and increases bone remodeling, favoring bone formation (Dempster et al., 1993, Jilka et al., 1999, Hodsman et al., 2005). Therefore, the decreased expression of osteocalcin in PTH-treated dwarf rats seems to conflict with the dramatic evidence for increased bone formation in these animals. However, other studies of the relationship between osteocalcin gene expression and PTH also show contrasting results. Noda (1988) used rat osteoblast-like osteosarcoma cells (ROS17/2.8) and observed that hPTH (1-34) increased osteocalcin mRNA levels in a dose-dependent manner and this effect would last up to 48h, with a peak at 24h (Noda et al., 1988). Sutherland (1994), also using an osteosarcoma cell line (SaOS-2), found that hPTH (1-34) had no significant effects on alkaline phosphatase (another indicator of bone formation, resulting from osteoblast activity) or osteocalcin mRNA levels (Sutherland et al., 1994). It is also intriguing that the lack of osteocalcin, by deleting both OG1 and OG2 genes in mice, leads to an increase in bone formation (Ducy et al.,
In this striking study, osteocalcin-deficient mice (osc\textsuperscript{m1}/osc\textsuperscript{m1}) were reported to be normal at birth, without skeletal malformations, viable and fertile, with no bone structural differences when compared to wild-type mice till 6 months of age. At this age, important changes started to develop: increased cortical thickness and density, accompanied by an increased amount of mineralized bone matrix, and increased width of the diaphysis. Cancellous bone was also increased in the mutant mice compared to their wild-type littermates, as was bone strength, evaluated by measurement of failure load as a biomechanical indicator. Histomorphometric analyses of fluorochrome labeling showed an increased bone formation rate, although osteoblast surface and osteoblast number were not increased.

On the other hand, there was an increase in osteoclast number and surface, and bone resorption occurred normally when these animals were ovariectomized. Most importantly, this study demonstrated that in osteocalcin null mice, bone formation rate increased without an increase in osteoblast number, indicating that the osteoblasts were depositing more bone matrix (Ducy et al., 1996), leading to the hypothesis that the synthesis of osteocalcin by the most mature osteoblasts would slow down the anabolic activity of these cells (Kronenberg, 1997, Yu and Chandrasekhar, 1997). PTH activated the osteocalcin promoter in osteosarcoma cell line (SaOS-2) cultures transiently, as very little induction was seen after 24 and 48h of treatment with PTH analogs (PTH 1-84, PTH 1-34, and PTH 1-31) (Yu and Chandrasekhar, 1997). Yet, we evaluated gene expression for osteocalcin at 14 days of PTH treatment, and we could have possibly exceeded the time for osteocalcin changes at the transcriptional level at that specific time point, although the final effects of increased bone formation were evident in pQCT,
histomorphometric and biomechanical analyses. We did not evaluate osteocalcin serum levels. A comparison of protein levels with gene expression in bone tissue may have allowed us to distinguish between the transcriptional and translational effects of PTH on osteocalcin.

PTH treatment did not induce any significant change in RANKL gene expression in dwarf rats, but a decrease was observed in Lewis rats, whereas no significant changes were seen in OPG gene expression in dwarf and Lewis rats. In an attempt to quantify how PTH could induce changes in RANKL and OPG gene expression at different stages of osteoblast differentiation, Huang (2004) made measurements at specific time-points in mouse primary bone marrow stromal cells. PTH induced minimal increases in RANKL gene expression from days 7 to 14, but a significant t difference was observed at day 21 (2 fold) and day 28 (3 fold). OPG gene expression was inhibited with PTH treatment, with a peak on day 14, and continued to show inhibitory effects at days 21 and 28, and these results were related to increased osteoclastogenesis (Huang et al., 2004). Suda (2004) also related PGE₂ to the inhibition of OPG gene expression (Suda et al., 2004), and RANKL mRNA levels were significantly increased in osteoporotic primary osteoblast cell cultures (Jurado et al., 2010).

Osterix, a transcription factor involved in osteoblast differentiation and bone formation, was downregulated by PGE₂, but not by PTH treatment. These results were puzzling, as Wang found that PTH (1-34) stimulated osterix mRNA expression in a dose-dependent manner, at low concentrations only (Wang et al., 2006), whereas in another study, PTH (1-34) treatment inhibited osterix mRNA levels and downregulated...
protein expression in osteosarcoma (UMR) cells and murine calvaria (Hong et al., 2009).

PTH and PGE\textsubscript{2} treatment significantly decreased sclerostin gene expression in dwarf rats. As mentioned before, PTH inhibits sclerostin expression \textit{in vitro} and \textit{in vivo}, with some different responses \textit{in vivo} that could be attributed to variations in time points at which sclerostin expression was analyzed after the last injection of PTH (Keller and Kneissel, 2005, Bellido, 2006). Sclerostin antagonizes Wnt signaling, a key signaling pathway in developmental processes, binding to the low density lipoprotein receptor-related protein, LRP5, resulting in decreased bone formation (Bezooijen et al., 2008).

Similar to sclerostin, LRP5 was discovered in studies of two rare diseases, high bone mass syndrome (HBM) and osteoporosis-pseudoglioma syndrome (OPPS). HBM is an autosomal dominant condition, characterized by gain-of-function of LRP5, leading to increased bone mass and formation, with a variable symptomatology, ranging from almost no symptoms to jaw enlargement, craniosynostosis, cranial nerve entrapment, and developmental delay. OPPS is a low bone mass disorder, with occurrence of juvenile-onset osteoporosis, fractures, and congenital or infancy-onset blindness, related to a loss-of-function mutation in the \textit{LRP5} gene (Balemans et al., 2008, Bezooijen et al., 2008, Ralston, 2008, Cui et al., 2011). The mechanisms involved in bone mass regulation by LRP5 are not fully understood, but \textit{in vitro} studies suggest a decreased inhibition of Wnt signaling by the antagonist proteins sclerostin and Dickkopf-1 (DKK1) (Li et al., 2005, Pangrazio et al., 2011, Cui et al., 2011). A complete lack of DKK1 is incompatible with life, but partial loss-of-function mutation in mice leads to
increased bone mass, whereas mice that overexpress DKK1 in osteoblasts have reduced bone mass (Balemans et al., 2008, Cui et al., 2011).

Sclerostin and DKK1 can both bind to LRP5; they act independently to antagonize Wnt signaling, and compete for binding to this receptor (Balemans et al., 2008). In view of these findings, we consider the effects of PTH treatment in LRP5 knockout mice. If no increase in bone formation occurs, the sclerostin downregulation mechanism of PTH action would be supported. On the other hand, if increased bone mass is demonstrated in LRP5 knockout mice treated with PTH, an alternative pathway involving one of the canonical Wnts reported to antagonize sclerostin, such as Wnt10b whose overexpression increases bone mass in mice, would then be considered (Bennett et al., 2005, Bennett et al., 2007, Bezooijen et al., 2008). PTH has been reported to induce bone anabolic effects in LRP5 knockout mice to the same extent as in wild-type mice (Sawakami et al., 2006, Iwaniec et al., 2007). Therefore, there must be some redundancy in the signaling pathways through which PTH stimulates bone formation.

Recently, PGE2 was found to decrease sclerostin expression in osteoblastic cell culture, without affecting DKK1 (Genetos et al., 2011), and in osteoblastic cells concomitantly exposed to mechanical strain (Galea et al., 2011). One of the limitations in the present study, relating mainly to PGE2, was the relatively short period of treatment. In other studies, in which PGE2 induced stronger bone anabolic effects, the period of treatment varied from 60 to 180 days of continuous daily injections, with doses between 3 and 6mg/kg body weight (Ito et al., 1993, Jee and Ma, 1997). However, Cui et al. (2001) reported that aged rats (20 months old) treated for only 10 days with the same dose of PGE2 as in the current study (3 mg/kg) exhibited a marked increase in
osteoblast surface, which was not observed in PGE$_2$-treated dwarf and Lewis rats (Cui et al., 2001). This discrepancy may be due to the much younger age of the rats (9 weeks old) in the current study.

In addition to the relatively short treatment period, we did not have enough groups of animals to obtain samples at different time points, and target the gene expression window for transcriptional changes in bone tissue. Transient changes in mRNA levels may have occurred before the cessation of treatment and euthanasia.

Conclusions

In our studies, PTH showed stronger bone anabolic effects than PGE$_2$, increasing bone mass and formation through downregulation of the sclerostin gene (SOST), even in IGF-I deficient rats. Sclerostin is a potent negative regulator of bone formation, and its downregulation may be responsible for the persistent anabolic effects of PTH treatment in dwarf rats. Since PTH strongly stimulated bone formation and augmented bone mass in growing, IGF-I deficient dwarf rats, this finding suggests that PTH would have anabolic effects in the osteopenic skeleton of juvenile humans with low serum levels of IGF-I, such as adolescents with anorexia nervosa.

Directions for Future Studies

Future studies are needed to compare the effects of PTH and PGE$_2$ treatments in aged and ovariectomized dwarf rats, and examine whether the osteopenic phenotype and responses to treatment would occur in the same manner. Further investigations would also be helpful to elucidate the mechanisms involved in the bone anabolic actions of PTH, such as the previously mentioned use of LRP5 knockout mice, and its evolution of fracture healing in relationship to the downregulation of sclerostin. It would
also be interesting to compare the evolution of fracture healing in GH/IGF-I deficient animals treated with PTH and PGE\textsubscript{2}.

The expression of a specific gene provides important information as to its biological role and state in cell metabolism; however, the regulation of protein abundance is not exclusively accomplished by regulation of mRNA (DeRisi et al., 1997). Therefore, we consider that proteomic analysis should be included to evaluate changes at the translational level, in addition to transcriptional mRNA levels.
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BIOGRAPHICAL SKETCH

Ana Cristina Ferreira Bassit was born in Santos, São Paulo, Brazil. She received her DVM degree from the College of Veterinary Medicine at University of São Paulo in 1985. In September, 2004 she achieved a master’s degree in experimental physiopathology, at the College of Medicine - University of São Paulo - FMUSP, Brazil. The research subject was the effect of extracorporeal shock wave therapy on bone healing after femur osteosynthesis with interlocking nails, an experimental study in dogs (Canis familiaris), performed at the College of Medicine at the University of São Paulo, Brazil.

In June 2007, she was selected by the J. William Fulbright Scholarship Board (FSB) for a Fulbright Student award in the United States for a Doctoral Program. In August 2007 she officially enrolled in the Graduate Program of the Department of Physiological Sciences at the College of Veterinary Medicine, University of Florida.

In May, 2008, she received a “Certificate for Outstanding Achievement” from the University of Florida International Center. In November 2008, and November 2009, she received the “Certificate of Academic Excellence” from the University of Florida International Center and the College of Veterinary Medicine.

On January 18th, 2011, she received a PhD Degree in Orthopedics - College of Medicine - University of São Paulo. This activity established an international academic cooperation between the Universities of Florida and São Paulo, so called “Agreement for Thesis under Co-Operation between Universities”. Faculty members of both Universities approved the dissertation with some of the results already obtained from the main project at University of Florida. The research subject was the effect of human parathyroid hormone fragment on bone metabolism, an experimental study in rats.
Her research interests include orthopedics, orthopedic surgery, fracture healing, bone growth factors, and bone research.