GROWTH HORMONE SIGNALING AND ACTION IN OSTEOBLASTS

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GROWTH HORMONE SIGNALING AND ACTION IN OSTEOBLASTS

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ABSTRACT

Growth hormone (GH) and insulin-like growth factor-1 (IGF-1) exert profound growth promoting actions during pre- and post-natal skeletal development. GH and IGF-1 appear to cause these anabolic actions by influencing a variety of effects on osteoblast proliferation, differentiation, and survival. However, because GH stimulates the production of IGF-1 from the liver and other GH-responsive peripheral tissues, including bone, the individual contributions of these two molecules to anabolic responses in bone remains poorly defined. In this dissertation research, I sought to distinguish the direct and indirect (IGF-1 dependent) GH actions on osteoblasts. In the first section of this thesis, I employed a genetic approach to disrupt the IGF-1 receptor (IGF-1R) specifically in osteoblasts, thereby eliminating its potential contribution to GH actions. In these studies, I demonstrated that even though direct actions of GH to reduce osteoblast apoptosis can be demonstrated \textit{in vitro}, the IGF-1R is required for the anabolic effects of GH on osteoblasts \textit{in vivo}. In the second part of this thesis, I employed another genetic mouse model to disrupt GHR specifically in osteoblasts. The results of these studies suggest that GHR is required for the full action of IGF-1 in osteoblasts \textit{in vitro}, but this deficit can be partially compensated \textit{in vivo} except in areas of the highest GHR concentration, e.g. cortex.
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As I complete this chapter of my life and career, I would like to thank everyone who has helped me to this point – I could have accomplished none of this on my own. Far too many people have supported me to name everyone individually, but hopefully, I have conveyed my thanks to them already…because most of them will probably never read this. To those who probably will read this – Mom, Dad, Paige, Tom – all of you have given me so much; I only hope that at some point, I can find a suitable way to repay my debt to you.
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## INTRODUCTION

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## MODE OF GROWTH HORMONE ACTION IN OSTEOBLASTS

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INTRODUCTION

Growth hormone (GH) and insulin-like growth factor-1 (IGF-1) exert profound growth promoting actions during pre- and post-natal skeletal development. GH and IGF-1 appear to cause these anabolic actions by influencing a variety of effects on osteoblast proliferation, differentiation, and survival. However, because GH stimulates the production of IGF-1 from the liver and other GH-responsive peripheral tissues, including bone, the individual contributions of these two molecules to anabolic responses in bone remains poorly defined. In this dissertation research, I sought to distinguish the direct and indirect (IGF-1 dependent) GH actions on osteoblasts. In the first section of this thesis, I employed a genetic approach to disrupt the IGF-1 receptor (IGF-1R) specifically in osteoblasts, thereby eliminating its potential contribution to GH actions. In these studies, I demonstrated that even though direct actions of GH to reduce osteoblast apoptosis can be demonstrated in vitro, the IGF-1R is required for the anabolic effects of GH on osteoblasts in vivo. In the second part of this thesis, I employed another genetic mouse model to disrupt GHR specifically in osteoblasts. The results of these studies suggest that GHR primarily serves to augment local IGF-1 levels in bone. In the following pages, I will provide a brief background on bone biology, the GH/IGF-1 axis, and discuss historical and current theories regarding GH/IGF-1 interaction, known actions of GH and IGF-1 in bone, and our study design.
The Formation and Remodeling of Bone

The formation of the mammalian skeleton occurs through a discrete stepwise process. Mesenchymal precursors first migrate to the future sites of bone, where they then condense. Following condensation, these precursors differentiate into chondrocytes or osteoblasts to form cartilage or bone, respectively, depending upon positional cues (1). Whether the condensing mesenchyme differentiates into osteoblasts or chondrocytes defines two different mechanisms responsible for the formation of the vertebrate skeleton – intramembranous and endochondral ossification.

The flat bones of the skull and clavicle are formed by intramembranous ossification. In this process, capillaries invade the mesenchymal zone, and mesenchymal precursors differentiate directly into osteoblasts which secrete osteoid matrix and subsequently mineralize it to form new bone. A more complex process called endochondral ossification accounts for the formation of the long bones and other bones of the skeleton. In this process, a cartilage template is formed which provides the framework for subsequent bone formation. Following mesenchymal condensation, mesenchymal precursors differentiate into chondrocytes and undergo a further process of stepwise, terminal differentiation. Two regions of chondrocytes first differentiate from resting chondrocytes to form a proliferative zone of chondrocytes. These proliferative chondrocytes then adopt a columnar organization and terminally differentiate into hypertrophic chondrocytes. The segregation of chondrocytes at varying stages of differentiation defines the two growth plates that separate the diaphysis (shaft of a long bone) from either epiphysis (head of the long bone) and dictates the primary (mid-diaphysis) and secondary (epiphyseal plate) ossification sites. These growth plates are responsible for the elongation of long bones.
through continued hypertrophic chondrocyte expansion. The cartilaginous extracellular matrix surrounding the terminally differentiated hypertrophic chondrocytes becomes calcified and these chondrocytes undergo apoptosis. Chondroclasts and preosteoclasts then degrade the cartilage to allow vascular invasion of the newly forming bone from the membrane of fibrous connective tissue surrounding the bone, the perichondrium. Osteogenic precursors are then recruited to this area, where they use the calcified cartilage template as a scaffold for the formation of trabecular bone. Osteoblasts also differentiate in the perichondrium to form the bone collar, serving as an outer structural support for the newly formed bone (2).

Maintenance of the mammalian skeleton is dependent upon the coordinated activities of three principle cell types: osteoblasts, osteoclasts, and osteocytes. Alterations in the functional activity of these cells results in dramatic changes in bone mass, morphology, and mineral content. When considered in a linear model, the bone remodeling cycle begins with a cycle of osteoclastic bone resorption. Into this cavity, osteoblasts are recruited from mesenchymal precursors. Mature osteoblasts fill the excavated bone cavities by secreting collagen and other matricellular proteins which compose the organic bone matrix. Osteoblasts mineralize bone matrix, during which time, some of them become entombed within their own matrix and further differentiate to become osteocytes (3).

While gross examination of bone might suggest it is a rather static organ, microscopic examination reveals it is one of the most dynamic tissues in the human body. The mammalian skeleton is subjected to a tremendous amount of mechanical strain on a daily basis, and its ability to support these loads requires that it be constantly remodeled to re-
pair microcracks that develop in both cortical and trabecular bone. This process is believed to be mediated by osteocytes functioning as a mechanostat (4). All mammals continue to remodel and form new bone throughout life. In adult humans, nearly 20% of the bone surface is undergoing remodeling at any given time, resulting in approximately 25% of trabecular and 3% of cortical bone being resorbed and replaced each year (5). Peak bone mass in humans occurs during the third decade of life. After this time, both cortical and trabecular bone mass declines as a result of increasing osteoclast activity and decline in both osteoblasts and osteocytes with age, resulting in unbalanced remodeling and consequent alterations in morphologic and material properties of the skeleton that lead to reduced bone strength (6).

A myriad of local growth factors, cytokines, and systemic hormones regulate osteogenesis and skeletal remodeling (7) (8). Bone formation, in particular, is dependent upon the proliferation, differentiation, and survival of osteoblasts. These processes are, in turn, regulated by locally produced autocrine/paracrine factors that include Wnts, Hedgehog and Notch, bone morphogenetic protein (BMP) families, transforming growth factor-β (TGF-β), IGF-1, fibroblast growth factor-2 (FGF-2), and interleukin-6 (IL-6) type cytokines (9). These autocrine/paracrine factors affect a number of transcription factors that regulate osteoblast differentiation, including Runx2, Osx, ATF4, and nuclear steroid hormone receptors (androgen/estrogen receptors) (10). As described below, GH and IGF-1 are important bone growth factors. While our current understanding of growth hormone may have begun over 50 years ago with experiments performed by Salmon and Daughaday examining the role of pituitary-regulated growth stimulating substances (11),
their complex interconnectedness has hindered our complete understanding of their action.

The Growth Hormone / Insulin-like Growth Factor-1 Axis

Growth hormone

GH is a member of a large family of cytokine peptides (12) that is produced and stored within the somatotroph cells of the anterior pituitary gland. GH exerts its effects by binding to the transmembrane GHR, which is expressed on the surface of most cells (13). Activation of the GHR initiates its growth promoting and metabolic effects in target tissues including increases in bone growth and mass, lipolysis, muscle growth, amino acid transport, and alterations in insulin sensitivity (14). Specifically, GH binds to the extracellular domain of GHR and initiates signaling through regulated interaction of signaling molecules with its ~350 residue cytoplasmic domain (15). The cytoplasmic domain of GHR however, is completely devoid of enzymatic activity, even as an active homodimer. Thus, GH binding triggers increased association with, and activation of, JAK2 – a cytoplasmic tyrosine kinase that is essential for most GH functions (16;17). Three downstream signaling systems activated in response to GH include signal transducers and activators of transcription (STAT – most notably for GH, STAT5b), phosphoinositide-3 kinase (PI3K), and extracellular signal-regulated kinase (ERK) (12;13). Activation of STAT5b by GH requires tyrosine phosphorylation of the cytoplasmic domain of GHR and results in transcription of such GH target genes as IGF-1 and the acid labile subunit (ALS) of the IGF binding protein 3 (IGFBP3) complex (18;19). Unlike STAT5b,
ERK induction does not require the entire cytoplasmic domain of GHR, but only effective coupling of JAK2 (20-23).

**Insulin-like growth factor-1**

Many, but not all of GH actions (24), are impacted through production of IGF-1 from liver and other peripheral tissues. IGF-1 inhibits GH secretion, and thereby exerts negative feedback to control GH production. IGF-1 is a small polypeptide that bears homology to pro-insulin and is produced by many cell types. It signals through the type 1 IGF-1R, a heterotetramer on the surface of many cells homologous to the insulin receptor, which possesses intrinsic tyrosine kinase activity in its cytoplasmic domain (25;26). Activation of IGF-1R results in signaling through SHC and insulin receptor substrate 1 (IRS-1) to activate PI3K and ERK, ultimately culminating in such functional responses as proliferation and anti-apoptosis (27). IGF-1 is also regulated by a family of IGF binding proteins which can serve as carrier proteins in the blood (e.g. IGFBP3) or locally to prevent/facilitate access to its cognate receptor (28). Some IGFBPs may even induce direct effects that are independent of IGF-1 (29). Binding of IGF-1 to one of the binding proteins forms what is referred to as a binary complex. These can further bind the acid labile subunit (ALS) to form the ternary complex, which restricts IGF-1 to the circulation and prolongs its half-life (30). GH is known to stimulate production of the ALS and IGFBP3, providing yet another level of complexity to the system.
Theories Regarding GH/IGF-1 Interaction

*The somatomedin hypothesis*

The interdependence of GH and IGF-1 actions (Fig. 1) has proved a significant obstacle in defining the discrete actions of GH. Evolutionarily, it is likely that the collaboration between these two hormones developed to allow appropriate tissue responses to GH, as well as to allow synergistic effects in certain contexts. Two main theories exist to describe this interaction, which serve as a conceptual framework for the development of our

![Schematic diagram of the GH/IGF-1 axis.](image)

**Figure 1. Schematic diagram of the GH/IGF-1 axis.**

The “somatomedin hypothesis,” as it was originally proposed in 1957 (11), postulated that somatotropin (GH) stimulated the hepatic secretion of somatomedin (IGF-1), which then mediated the actions of somatotropin in responsive tissues. Minor revision to this theory came in 1980, when explants of fetal mouse tissues cultured in serum-free media showed higher levels of somatomedin-C (IGF-1) than extracts of those tissues themselves (31). IGF-1 was subsequently shown to be produced by a number of peripheral tissues, providing a means for IGF-1 to exert autocrine/paracrine effects. Evidence
from genetically altered mice has led to modifications of this hypothesis. Liver-specific IGF-1 ablation, for example, reduced serum IGF-1 levels by 75% without an effect on normal growth (32;33). Further reducing circulating IGF-1 levels (85-90% reduction) by knocking out the ALS component of the IGF-1/IGFBP3/ALS ternary complex in the LID/ALSKO mouse resulted in 30% reduction in postnatal growth accompanied by retarded bone growth and acquisition (34). Taken together, these studies suggest that while circulating IGF-1 may play a minor role in longitudinal bone growth and volume, autocrine/paracrine-derived IGF-1 appears to contribute more significantly to normal postnatal growth.

The dual effector hypothesis

An alternative model of GH/IGF-1 interaction was proposed by Green and co-workers in 1985 (35). Using preadipocytes as a model, GH was postulated to stimulate adipocyte differentiation, while IGF-1 stimulated their clonal expansion. This theory was extended to the growth plate by Isaksson’s group, who proposed that GH acted directly at the germinal zone to stimulate differentiation of chondrocytes (36). The induction of local synthesis of IGF-1 by GH was thought to stimulate the clonal expansion of chondrocyte columns in an autocrine/paracrine manner (37). In support of the dual effector theory, disruption of both GHR and IGF-1 genes in mice resulted in >80% growth retardation – a significantly greater response than disruption of either gene individually (38). Interestingly, IGF-1 null mice, which exhibit increased circulating GH levels due to loss of feedback inhibition, have an enlarged germinal zone and reduced chondrocyte hypertrophy (38), whereas mice lacking GHR exhibit both defective chondrocyte generation
and hypertrophy (39). Such evidence supports the dual effector concept and suggests that GH and IGF-1 serve distinct functions in cartilage.

Evidence for synergism between GH and IGF-1

In contrast to these more familiar models, recent studies suggest the possibility that under certain conditions, GH and IGF-1 can act in a collaborative or even synergistic fashion. Treatment of serum starved 3T3-F442A cells with both GH and IGF-1 induced c-fos mRNA levels greater than their summed individual responses (40). In GHR expressing melanocytes, GH alone had no effect on proliferation and cell number whereas IGF-1 alone increased cell numbers, and the combination of both amplified this response by 50% (41). GH/IGF-1 synergy was also observed in cultured human osteoblasts by Langdahl and colleagues (42). These investigators showed that GH pre-treatment increased osteoblast sensitivity to IGFs, similar to the observations cited above in preadipocytes and prechondrocytes. Studies from Frank and colleagues, suggest that in 3T3-F442A cells, GH/IGF-1 synergy may involve a physical interaction of IGF-1R with the GHR/JAK2 complex (43).

Evidence also exists which suggests that the interaction of GH and IGF-1 signaling pathways extends beyond the receptor level. Both GHR and IGF-1R are known to cluster in caveolae (44;45), which are 50-100nm invaginations of the plasma membrane with high cholesterol content and a caveolin coat found on a wide variety of cell types (46-48). Caveolae are believed to be a specialized form of lipid raft that is anchored to the plasma membrane by the actin cytoskeleton and involved in recruitment of components for intracellular signaling, as well as endocytosis (48). Given the significant over-
lap in the signaling cascades activated by GHR and IGF-1R, it is reasonable to speculate that positioning of these receptors together could enable shared signaling components. One such common signaling component is Src homology 2 (SH2) B adaptor protein (SH2-B). SH2-B has been shown to associate with JAK1, JAK2, PDGFR (49), IRS-1 and IRS-2 (50), thus linking it to GHR and IGF-1R signaling. It is known to bind and enhance the activity of JAK2 following GH stimulation and autophosphorylation of JAK2, acting as a positive feedback loop. It has also been shown to associate with JAK2 in an inactive state (49). One could envision a GHR-JAK2-SH2-B complex that would provide positive feedback for IGF-1 signals in the close proximity of the caveolae. In this model, IGF-1R autophosphorylation and activation of IRS-1/2 would lead to SH2-B activation, and in turn, JAK2. JAK2 could then activate additional IRS1/2 or other arms of IGF-1 signaling like SHC-GRB2-SOS which would ultimately culminate in MAPK activation.

GH and IGF-1 Action in Bone

*Actions of IGF-1 in osteoblasts*

The ability of IGF-1 to induce osteoblast proliferation is well known. In MC3T3 osteoblast-like cells, proliferative effects of IGF-1 are associated with increased levels of c-fos mRNA (51). IGF-1 is also recognized as a key survival factor for osteoblasts (52). In addition, IGF-1 exerts a number of effects on differentiated osteoblasts. Early in the differentiation of fetal rat calvarial osteoblasts in vitro, IGF-1 production increases, possibly serving to promote progression from preosteoblast to mature osteoblast. As the differentiated phenotype appears, IGF-1 expression declines (53). IGF-1 expression rises
again however, as a second wave accompanies osteoblast maturation, matrix synthesis and mineralization. This IGF-1 production may serve to augment synthesis of type I collagen and inhibit collagen degradation in differentiated fetal rat osteoblasts (54,55).

**Effects of IGF-1 on bone in vivo**

Insights into IGF-1 actions on the skeleton have come from studies in genetically altered mice. Mice globally deficient in IGF-1 mentioned above, exhibit delayed ossification and reduced birth weight (~60% of normal). Mice that survive to adulthood maintain a reduced growth rate throughout life and are ~30% of normal size at the age of 2 months with delayed bone development (56). Distinguishing discrete effects of IGF-1 on bone using this model is problematic because of the involvement of a number of other organ systems affected by the loss of IGF-1, e.g. reproductive organs. Tissue-specific model systems utilizing Cre-loxP recombination strategies circumvent this issue and have allowed more definitive examination of the role of IGF-1 in bone development. Targeted overexpression of IGF-1 in osteoblasts accelerates new bone formation as well as the rate at which that bone is mineralized (57). Conversely, osteoblast-specific disruption of IGF-1R markedly impairs mineral apposition rate and increases mineralization lag time (58). Interestingly, the effects of these genetic manipulations of IGF-1 are most pronounced during the pubertal growth spurt, likely owing to an interaction of IGF-1 and the sex steroids (59).

A number of humans bearing mutations in various components of the IGF-1 pathway also highlight the importance of IGF-1 in normal bone formation. A 15 year old patient with homozygous deletion of exons 4 and 5 of the *IGF1* gene displayed severe
intrauterine growth retardation and postnatal growth failure, including delayed bone development (60). The effects of IGF-1 in bone are again confounded by a number of other affected organ systems in this patient. Patients with mutations in the \textit{IGFALS} gene, which encodes the acid labile subunit of the IGF-1/IGFBP3/ALS ternary complex, displayed moderate postnatal growth retardation with an apparent delay in bone maturation as indicated by dual-energy x-ray absorptiometry (DEXA) analysis (61). These patients had undetectable levels of ALS and low IGF-1 in the presence of normal GH levels. Here again, pinning down the direct actions of IGF-1 in bone is difficult due to concomitant hyperinsulinemia. Regardless of the many confounding variables affecting these patients carrying mutations in the IGF-1 pathway, taken together, they clearly support a role for IGF-1 in pre- and postnatal bone formation.

\textit{Actions of GH on osteoblasts}

Because of the interdependence of GH and IGF-1 actions, it has been difficult to define the direct effects of GH on osteoblasts. High-affinity GHRs are expressed on rat osteoblast-like UMR106 osteosarcoma cells (62) as well as human (63;64) and mouse (65;66) primary osteoblasts. GH can induce osteoblast proliferation in culture (63), but whether this effect requires IGF-1 production is unclear. Administration of IGF-1 to GH deficient rats yields sub-optimal growth (67), but whether such effects are mediated by the osteoblast is unknown.
Effects of GH on bone in vivo

Studies of mice globally deficient in GH or IGF-1 have provided some evidence that these two hormones may act independently. Mohan and colleagues reported severely impaired postnatal femoral length, size and BMD in IGF-1 globally deficient and GH globally deficient (lit/lit) mice, but a larger defect in the IGF-1 deficient mice (40-55% reduction vs. 25-32% reduction in lit/lit) suggests independent functions of GH and IGF-1 (68). Moreover, GH was able to significantly increase trabecular bone formation rates in IGF-1 null mice (69). Interpretation of these results is confounded however, by severe growth disturbances in other organ systems of these mice, as well as problems with reproductive hormone status. Despite these shortcomings, these results are compatible with the idea that GH and IGF-1 exert overlapping and independent functions. Mice overexpressing the IGF inhibitory binding protein IGFBP4 in osteoblasts, a model where IGF-1 would presumably be sequestered from bone target cells, bone volume was only 2.8% (70) as compared to 11.4% seen in mice overexpressing IGF-1 in osteoblasts (maximal IGF-1 access) (57). This four-fold difference in bone volume attests to the magnitude of the overall effect of IGF-1 on postnatal bone acquisition. It is likely that GH contributes to this overall bone anabolic effect as well, but its contribution could not be examined experimentally because of the inability to distinguish the actions of GH from the downstream effects of IGF-1 production.

In summary, sophisticated methods of manipulating the mouse genome have provided powerful new tools for the study of locally active growth factors in vivo. In particular, the Cre/LoxP system (71) allows targeted disruption of mouse genes in a tissue specific fashion, thus eliminating complications of global knockout models mentioned
above. Based on this strategy, we have developed mouse models with conditional disruption of either IGF-1R or GHR in osteoblasts. In the first section of this thesis, we use osteoblast specific disruption of the IGF-1R to define direct actions of GH on osteoblasts. We then use the conditional GHR disruption model to define the role of GHR in osteoblast function, described in the second half of this thesis.
MODE OF GROWTH HORMONE ACTION IN OSTEOBLASTS

by

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Format adapted and errata corrected for dissertation
Abstract

Growth hormone (GH) affects bone size and mass in part through stimulating IGF-1 production in liver and bone. Whether GH acts independent of IGF-1 in bone remains unclear. To define the mode of GH action in bone, we have used a Cre/LoxP system in which the type 1 IGF-1 receptor (Igf1r) has been disrupted specifically in osteoblasts in vitro and in vivo. Calvarial osteoblasts from mice homozygous for the floxed IGF-1R allele (IGF-1R\textsuperscript{flox/flox}) were infected with adenoviral vectors expressing Cre (Ad-Cre). Disruption of IGF-1R mRNA (>90%) was accompanied by near elimination of IGF-1R protein but retention of GHR protein. GH induced STAT5 activation was consistently greater in osteoblasts with an intact IGF-1R. Osteoblasts lacking IGF-1R retained GH induced ERK and Akt phosphorylation and GH stimulated IGF-1 and IGFBP-3 mRNA expression. GH induced osteoblast proliferation was abolished by Cre-mediated disruption of the IGF-1R or co-incubation of cells with an IGF-1 neutralizing antibody. By contrast, GH inhibited apoptosis in osteoblasts lacking the IGF-1R. To examine the effects of GH on osteoblasts in vivo, mice wild-type for the IGF-1R treated with GH subcutaneously for 7 days showed a doubling in the number of osteoblasts lining trabecular bone, whereas osteoblast numbers in similarly treated mice lacking the IGF-1R in osteoblasts were not significantly affected. These results indicate that although direct IGF-1R independent actions of GH on osteoblast apoptosis can be demonstrated in vitro, IGF-1R is required for anabolic effects of GH in osteoblasts in vivo.
Introduction

The process of osteogenesis and remodeling of the skeleton is orchestrated by a constellation of local growth factors, cytokines, and systemic hormones (1;2), of which, GH and IGF-1 are key components. GH belongs to a family of cytokine peptides (3) and is produced and stored by somatotroph cells within the anterior pituitary. GH actions are mediated by binding to the transmembrane GHR, thereby triggering increased association with and activation of JAKs (4-6) to activate STATs (7-15), PI3K/Akt (16;17), and ERK (18-20). Growth hormone exerts many, but not all (21), of its effects by stimulation of IGF-1 from liver and peripheral tissues. IGF-1 is a small polypeptide with homology to pro-insulin that is produced by a number of cell types. IGF-1 signals via the type 1 IGF-1 receptor (IGF-1R), engaging ERK and PI3K pathways through SHC and insulin receptor substrates-1 and 2 (IRS-1/2) (22;23). The effects of IGF-1 on bone have been well documented. IGF-1 has been shown to induce proliferation of MC3T3 osteoblast-like cells (24) and is an important survival factor for many mammalian cell types including osteoblasts. IGF-1 production increases during the initial phases of fetal rat calvarial osteoblast differentiation in vitro and then declines with the appearance of a differentiated phenotype (25). This is followed by a second wave of IGF-1 production that occurs with matrix synthesis and mineralization that may account for the ability of IGF-1 to augment synthesis of type I collagen and inhibit collagen degradation in differentiated fetal rat osteoblasts (26). In mice, targeted overexpression of IGF-1 accelerates new bone formation as well as increases the rate at which matrix is mineralized (27), while osteoblast-specific disruption of the IGF-1R causes markedly impaired mineral apposition rate and increased mineralization lag time (28).
In contrast to the large volume of literature on IGF-1 actions in osteoblasts, little is known regarding direct GH effects in this cell type. High affinity GH receptors are found on rat osteoblast-like UMR106 osteosarcoma cells (29) and human (30) and mouse (31-33) primary cultured osteoblasts. GH can induce osteoblast proliferation in cell culture (30), but whether this action requires IGF-1 production remains uncertain. Genetic mouse models globally deficient in IGF-1 or GH provide circumstantial evidence for independent functions of GH and IGF-1 on osteoblast mediated bone formation. For example, Mohan and colleagues reported that mice globally deficient in either IGF-1 or GH (lit/lit) had markedly impaired postnatal BMD, but that the defect was more severe in IGF-1 nulls (34). However, severe disturbances of overall growth of these mice and problems with reproductive hormone status confound interpretation of these previous models.

Because of the intimate relationship between GH and IGF-1, it has been difficult to precisely define discrete actions of each of these factors. Here we used a Cre-loxP method to selectively disrupt the IGF-1R in osteoblasts. Results from this model suggest that while IGF-1R-independent actions of GH to inhibit osteoblast apoptosis can be demonstrated in vitro, IGF-1R is required for normal GH action in osteoblasts in vivo, presumably through its ability to stimulate the production of IGF-1.
Experimental Procedures

Materials

Cell culture media, $\alpha$-minimal essential medium ($\alpha$MEM), was obtained from Cellgro-Mediatech (Herdon, VA) and fetal bovine serum (FBS) was from Gibco (Gaithersburg, MD). Bovine GH was obtained from the National Hormone and Peptide Program – Monsanto (Torrance, CA) and stored in 200X aliquots for single use. Human IGF-1 was obtained from GroPep (Theberton, SA, Australia) and stored in 1000X aliquots for single use. PDGF-BB was obtained from Sigma and stored in 430X aliquots for single use. Antibodies used for immunoblotting included anti-IGF-1R $\beta$ subunit (C-20), anti-BAX (N-20), anti-$\beta$-actin (C4) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-phospho-STAT5 (Tyr 694), anti-STAT5, anti-phospho-Akt (Ser 473), anti-Akt, anti-phospho-ERK (Thr202/Tyr204), anti-ERK from Cell Signaling Technology (Danvers, MA); anti-GHR (AL47) was a generous gift from Dr. Stuart Frank. IGF-1 neutralizing antibody (Clone 126002) was obtained from R&D Systems (Minneapolis, MN). Horseradish-peroxidase conjugated rabbit and mouse secondary antibodies were obtained from Pierce Biotechnology (Rockford, IL). Polyvinylidene difluoride (PVDF) membrane, Laemmli sample buffer and other electrophoresis supplies were from Bio-Rad. Assay kits for flow cytometry analysis of cell proliferation and apoptosis were purchased from BD Pharmingen (San Jose, CA). All other reagents not specified here were purchased from Sigma.
Osteoblast isolation and culture

Osteoblasts were isolated from calvaria of newborn $\text{Igf1r}^{\text{flox/flox}}$ mice or ROSA26 reporter mice by serial digestion in 1.8 mg/ml collagenase type I (Worthington, Lakewood, NJ) solution. Calvaria were digested in 10 ml of digestion solution for 15 min at 37 °C with constant agitation. The digestion solution was collected, and digestion was repeated with fresh digestion solution an additional four times. Digestions 3–5 (containing the osteoblasts) were pooled together, centrifuged, washed with αMEM containing 10% FBS, 1% pen/strep, and plated overnight at 37 °C in a humidified incubator supplied with 5% CO₂. For in vitro deletion of the IGF-1R, osteoblasts containing floxed IGF-1R alleles were cultured to be 70% confluent and then, in the absence of serum, were infected with adenovirus encoding Cre recombinase (Ad-Cre) (Vector Biolabs, Philadelphia, PA) at a titer of 100 multiplicity of infection (moi). Infection with 100 moi of adenovirus encoding green fluorescent protein (Ad-GFP) (Vector Biolabs) was used as control. After 1 hr, culture medium containing 10% FBS was added and the cells were allowed to recover for the next 48 hr. Greater than 90% IGF-1R deletion was confirmed for every infection by quantitative real-time PCR.

Animal studies

Female control ($\text{Igf1r}^{\text{flox/flox}}$) and Δ$\text{Igf1r}$ mice ($\text{Igf1r}^{\text{flox/flox Cre+}}$) 3 weeks of age were treated subcutaneously with GH (3 ug/g body weight/day) or vehicle (0.01% NaHCO₃ in PBS) for 7 consecutive days. During this period, all mice were also injected intraperitoneally with 100 uL of a 1% calcein solution (to assess dynamic histomorphometric parameters) on a split dose schedule leaving three days between the first and
second dose, and 2 days following the second dose before sacrifice. Distal femora were fixed in 100% ethanol, embedded and sectioned. Five serial sections were stained using the Masson-Goldner trichrome technique, and five more serial sections were left unstained for fluorescent microscopy. Static and dynamic parameters of bone structure and formation were measured using the OsteoMeasure™ system (OsteoMetrics). Serum was also collected upon sacrifice of the animals, and serum IGF-1 measurements were performed at MERCORE Laboratory (Bangor, ME).

**LacZ staining**

Paraformaldehyde fixed samples of primary osteoblasts from ROSA26 reporter mice were washed three times for 5 min in lacZ wash buffer (2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% Nonidet-P40, 5 mM EGTA in PBS). Staining was carried out in lacZ staining buffer (1 mg/ml X-gal, 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide in lacZ wash buffer) at 37 °C for 4 hr to overnight, with shaking and protection from light. After staining, samples were rinsed with PBS, post-fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 10 min, rinsed twice with PBS, and then twice with 70% ethanol prior to storage in 70% ethanol at 4 °C.

**Cell lysis and immunoblotting analysis**

For signaling experiments, ΔIGF-1R and control cells were cultured in 10% FBS αMEM to 90% confluence and then serum starved in 0.1% BSA containing αMEM for 24 hr to reduce cellular activity to quiescent levels. At the end of the study, the cells
were washed twice with ice cold phosphate-buffered saline (PBS) and resuspended in lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM MgCl₂, 1 mM EDTA, 1% Triton X-100, and 10% glycerol). Protease and phosphatase inhibitors (Sigma) were added to the lysis buffer. The cell lysates were homogenized by needle aspiration and protein concentration was measured by Bradford protein assay (Bio-Rad). For immunoblotting of whole cell lysates, equal amounts of protein (10 or 20 µg/lane) were solubilized in Laemmli sample buffer and loaded onto a mini-SDS-PAGE system. Following electrophoresis, proteins were transferred to a PVDF membrane using a Bio-Rad semi-dry transfer system. Protein transfer efficiency was verified using prestained protein markers. Membranes were then blocked with 5% non-fat dry milk for 1 hr at room temperature and subsequently incubated overnight at 4°C with antibodies directed against the protein of interest. Signals were detected using a horseradish peroxidase-conjugated secondary antibody and bound antibodies were visualized using the Supersignal West Femto Substrate (Pierce). Western blot photographic results were scanned with a Canon flatbed scanner. The relative levels of proteins of interest were then determined by measuring the density of the corresponding bands with AlphaEaseFC™ software (Alpha Innotech). All values were averages of at least three experiments and were normalized to the protein expression of a normalization protein.

**Quantitative real-time PCR**

Total RNA was extracted from cells using the TRIzol® method as recommended by the manufacturer (Invitrogen). The RNA concentration was estimated spectrophotometrically and only pure RNA (A260:A280 ratio ≥ 1.8) was used for further analysis.
First strand cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad). The cDNA was amplified in the Opticon Continuous Fluorescent Detector (MJ Research, Waltham, MA) using IQ™ SYBR Green supermix (Bio-Rad) and sequence specific primers. PCR reactions were performed in triplicate for each cDNA, averaged, and normalized to endogenous β-actin reference transcripts. Primer sequences used were as follows: IGF-1R: F5’-TTGTGTGTGTTTCGTCGGGTGTG-3’, R5’-ATGTGCCCAAGTGTGTGCG-3’; IGF-1: F5’-GCTCTTCAGTTCTGTGTGGAGAC-3’, R5’-TTGGGCTGTCAGTGTGGCGC-3’; IGFBP3: F5’-ACAGACACACAGAACCACCCTTCTCCTC-3’, R5’-TGCTTTCTGCTTTGGAAGGGGC-3’; β-actin: F5’-ACCTCCTACAATGAGCTGC-3’, R5’-TGCCAATAGTGATGACCT-3’.

Osteoblast proliferation assays

For the Promega CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega), the ΔIGF-1R and control osteoblasts were plated in 96-well plates at low cell density (3 X 10³ cells per well) and cultured in αMEM with 1% FBS for 24 hours to arrest the cells in G0 phase. The cells were then restimulated with 40 ng/mL PDGF-BB, 500 ng/mL GH or 100 ng/mL IGF-1 for 48 hours. For IGF-1 neutralizing antibody experiments, the manufacturer indicated ND50 dose of IGF-1 neutralizing antibody for 100ug/mL IGF-1 (10ug/mL anti-IGF-1) was co-incubated with GH or IGF-1. CellTiter reagent was then added to each well per manufacturer’s instruction and plate results were read spectrophotometrically 1.5 hours later at 490nm (Thermo Labsystems OpsysMR and Revelation Quicklink software). For the flow cytometric analysis of BrdU incorporation, the ΔIGF-1R and control osteoblasts were plated in 6-well plates at low cell density (9 X
10^4 cells per well) and cultured in αMEM with 1% FBS for 24 hours to arrest the cells in G0 phase. The cells were then restimulated with 500ng/mL GH or 100ng/mL IGF-1 for 48 hours. For proliferation analysis of the cells, 10 μM BrdU was added to the medium 12 hr before harvesting cells. The cells were then stained for anti-BrdU-APC and 7-AAD for proliferation and analyzed by FACS Calibur (Becton-Dickson). 20,000 events were collected for each sample and results were analyzed with WinMDI version 2.8.

_Osteoblast apoptosis assays_

Control and ΔIGF-1R osteoblasts were grown to confluent monolayers in 6-well plates – on cover slips for TUNEL – and cultured in αMEM with 1% FBS containing 500ng/mL GH for 24 hours prior to induction of apoptosis with 8ng/mL staurosporine for 24 hours. For BAX analysis, protein lysates were harvested and subjected to SDS-PAGE as described above. For TUNEL staining, cover slips were fixed and stained according to manufacturer instructions for the In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science). TUNEL positive cell numbers were determined by direct counting.

_Statistical Analysis_

All statistical analyses were performed using the Microsoft Excel data analysis program for ANOVA or Student’s t-test analysis with an assigned significance level of 0.05 (α). All experiments were repeated at least three times unless otherwise stated. Values were expressed as a mean ± S.E.
Results

Disruption of IGF-1R in osteoblasts

To establish a system for Cre-mediated gene excision of the IGF-1R in vitro, primary osteoblasts from calvaria of ROSA26 reporter mice were infected with adenoviral Cre (AdCre) or GFP control (AdGFP) vectors and stained by X-gal staining. In this model system, Cre excises a floxed neomycin cassette within the LacZ gene allowing expression and positive blue staining in cells in which Cre is expressed (35). A 100 moi dose of AdCre resulted in nearly 100% excision as shown by the presence of blue staining cells (Fig. 1A). We next assessed AdCre mediated deletion in the \textit{Igf1r} locus by infecting primary osteoblasts of calvaria from mice carrying the floxed \textit{Igf1r} allele. Real time PCR analysis of IGF-1R mRNA expression (Fig. 1B), as well as immunoblotting for IGF-1R (Fig. 1C), indicated >90% removal of IGF-1R at a 100 moi dose of AdCre. The level of GHR as assessed by immunoblotting (Fig. 1D) was not affected by the removal of IGF-1R.

To assess the effect of IGF-1R disruption on the responsivity of osteoblasts to other mitogenic stimuli, calvarial osteoblasts from \textit{Igf1r} floxed mice were treated with platelet-derived growth factor (PDGF), a potent mitogen for osteoblasts (36). Immunoblotting for phospho-Erk (Fig. 1E) in extracts of cells treated for 10 minutes showed equivalent phosphorylation in AdGFP and AdCre treated osteoblasts. PDGF induced similar BrdU uptake in AdGFP and AdCre treated cells (Fig. 1F) indicating that those osteoblasts lacking IGF-1R retained responsiveness to other osteoblastic mitogens.
GHR signaling in the absence of IGF-1R

To examine the effect of IGF-1R on GH signaling, calvarial osteoblasts from mice carrying the floxed \(Igf1r\) allele were infected with AdGFP (hereafter referred to as control) or AdCre (hereafter referred to as \(\Delta IGF-1R\)) and treated with GH or IGF-1. Immunoblotting for phospho-STAT5 (Fig. 2A) showed slightly greater activation in control cells compared to \(\Delta IGF-1R\) cells. Signaling through Akt and ERK (Fig. 2B and 2C, respectively) was assessed by immunoblot in the same manner. IGF-1 induced phospho-ERK was reduced to basal levels following removal of the IGF-1R (Fig. 2C; lane 3 vs. lane 6) while GH induced ERK activation actually increased modestly in the absence of the IGF-1R (Fig. 2C; lane 2 vs. lane 5). Similar results were seen for Akt activation (Fig. 2B). In osteoblasts with an intact IGF-1R, GH induced only modest Akt activation compared to the robust induction seen following IGF-1 treatment. Following removal of IGF-1R however, IGF-1 induced Akt activation was nearly abolished (Fig. 2B; lane 3 vs. lane 6) while GH retained levels of Akt activation similar to those seen in the cells containing IGF-1R (Fig 2B; lane 2 vs. lane 5).

IGF-1R is not required for GH stimulated IGF-1 and IGFBP-3 mRNA production

We next determined the effects of loss of IGF-1R on the transcription of established GH target genes IGF-1 and IGFBP-3. GH stimulated IGF-1 mRNA by two-fold over basal levels in control osteoblasts (Fig. 3A). Interestingly, osteoblasts lacking IGF-1R had basal levels of IGF-1 mRNA comparable to those of stimulated control cells, but GH treatment still showed further significant increase in IGF-1 mRNA. Similar results were seen for IGFBP3 mRNA (Fig. 3B).
GH inhibits osteoblast apoptosis in vitro in the absence of IGF-1R

To determine the effect of GH on osteoblast apoptosis, control and ΔIGF-1R osteoblasts were pretreated with GH or vehicle in media containing 1% FBS before the induction of apoptosis with staurosporine. Apoptosis was measured by immunoblotting for BAX, a pro-apoptotic protein and TUNEL staining (Fig. 4A and 4B, respectively). In the presence of IGF-1R, GH reduced staurosporine induced apoptosis as indicated by reduced BAX levels and decreased TUNEL positive cells. Following removal of IGF-1R, GH still modestly attenuated the apoptotic effects of staurosporine, although the basal apoptotic rates were increased in osteoblasts lacking the IGF-1R; likely due to the loss of autocrine IGF-1 survival signals since osteoblasts express both IGF-1 and IGF-1R.

GH requires IGF-1R to induce osteoblast proliferation in vitro

To assess the effect of IGF-1R on GH induced osteoblast proliferation, control and ΔIGF-1R osteoblasts were serum starved for 24 hours and then treated with GH or IGF-1. Proliferation was assessed by flow cytometry for BrdU incorporation (Fig. 5A) as well as Promega CellTiter 96® AQeuous Cell Proliferation Assay (Fig. 5B). In control osteoblasts, GH and IGF-1 induced osteoblast proliferation by both assays, whereas GH failed to increase proliferation in ΔIGF-1R osteoblasts.

This result suggests that GH induces proliferation in osteoblasts primarily by increasing IGF-1 production. To test this idea, wild type osteoblasts treated with GH or IGF-1 following serum starvation were cultured in the presence of an anti-IGF-1 neutralizing antibody. As shown in Fig. 5C, the proliferative effects of GH were ablated in the
presence of the IGF-1 neutralizing antibody, demonstrating that GH promotes osteoblast proliferation through the production of IGF-1.

**GH requires the IGF-1R to induce osteoblast proliferation in vivo**

In previous studies (28), we found that 3 week old $\Delta Igf1r$ mice had significantly fewer osteoblasts and reduced trabecular bone formation rate compared to controls. To evaluate the effect of GH on osteoblasts in vivo, $\Delta Igf1r$ mice and control littermates were treated subcutaneously with 3µg/g bw/day GH for 7 days. Serum IGF-1 measurements showed equivalent fold increases in serum IGF-1 levels in both control and knockout mice (Fig. 6E). Treatment of control mice with GH (Fig. 6B – arrows & Fig. 6F) nearly doubled osteoblast number compared to vehicle treatment (Fig. 6A & 6F). Osteoblast number (Fig. 6C & 6F) and bone formation rate (Fig. 6G) were decreased approximately 50% in the knockout animals, in agreement with our previous characterization of these mice. In contrast to control mice however, GH treatment of mice lacking IGF-1R in osteoblasts had no effect on osteoblast number (Fig. 6D & 6F) or bone formation rate (Fig. 6G).
Discussion

GH profoundly influences bone growth and mass, but despite decades of work, the cellular targets and mechanisms of action of GH in bone remain unclear. The principal difficulty in studying GH action is the fact that GH stimulates IGF-1 production making it virtually impossible to distinguish actions due to GH versus those resulting indirectly through IGF-1. A number of previous studies have provided circumstantial evidence that GH directly influences skeletal development and bone formation independent of IGF-1. For example, administration of IGF-1 to GH-deficient rats results in suboptimal growth (37). Further, mice globally deficient in either IGF-1 or GH (lit/lit) had a markedly impaired postnatal BMD, but that defect was more severe in the IGF-1 nulls (34). Moreover, it has been reported that GH administration significantly increased trabecular bone formation rates in IGF-1 null mice (38). Taken together, these studies suggest that the GH and IGF-1 signaling pathways serve both independent and overlapping functions in the skeleton. However, severe disturbances in overall growth of these mice and problems with reproductive hormone status limit conclusions about direct effects of GH in bone.

In these studies, we used a Cre-LoxP technique to selectively disrupt the IGF-1R in osteoblasts, enabling us to examine the actions of GH on this cell type in the absence of confounding effects brought about by IGF-1R signaling. In osteoblasts with an intact IGF-1R, GH effectively coupled to its downstream effector STAT5 and stimulated activation of both ERK and Akt pathways. However, removal of IGF-1R caused a modest but significant attenuation of GH induced STAT5 activation, suggesting that IGF-1R and GHR might normally interact in cells that express both receptors. Such collaboration be-
tween GHR and IGF-1R appears to occur in 3T3-F442A preadipocyte cells and might account for synergistic activation of c-fos in response to combined GH and IGF-1 treatment (39). Indeed, it has been proposed that synergy could occur in these cells through physical interaction of GHR and IGF-1R at the level of JAK2 (40). Whether, or to what extent, such a mechanism exists in osteoblasts is unknown and is currently under further investigation in our laboratory.

Despite the potential for interaction of GH and IGF-1R signaling just described, disruption of IGF-1R in osteoblasts in vitro did not abolish the ability of GH to stimulate its target genes, IGF-1 and IGFBP-3, and block apoptosis. Interestingly, osteoblasts lacking IGF-1R had elevated basal levels of IGF-1 and IGFBP-3 mRNA, possibly due to loss of negative feedback signaling, and increased basal apoptotic frequency. In addition, previous studies by Morales et. al. in osteoblast-like UMR 106 cells also showed that GH inhibited apoptosis (41). Functional STAT5 response elements have been described in the BCL-xL promoter (42), providing a potential mechanism whereby GH might block osteoblast apoptosis in the absence of a functional IGF-1R. These results clearly show that GH signaling through its receptor in osteoblasts can exert direct actions in osteoblasts independent of IGF-1R.

By contrast, the growth promoting actions of GH appear to require IGF-1R signaling since they are eliminated in osteoblasts lacking the IGF-1R. Studies in osteoblasts from human trabecular bone explants and SaOS-2 osteosarcoma cells (33), as well as human bone marrow stromal osteoblast-like cells (43), have shown that GH induces proliferation in all of these cell types. However, the proliferative effects of GH on the SaOS-2 cells are abolished in the presence of an IGF-1 neutralizing antibody (33), in agreement
with our findings. Most importantly, our studies show that in vivo administration of GH increased the number of osteoblasts only in mice with an intact IGF-1R. This finding demonstrates that, although GH can prevent osteoblast apoptosis in vitro independent of IGF-1R, the in vivo effects of GH on osteoblast number in vivo are likely to be mediated indirectly via IGF-1 arriving from the circulation or produced locally in bone. We think it unlikely that the anti-apoptotic effect of GH shown in vitro would have a significant impact on osteoblast number in vivo, at least under the short term treatment protocol used in this study. Analysis of GH effects beyond one week in this model would be complicated by the transient nature of the bone phenotype of this mouse; decreased osteoblast numbers seen in ∆Igf1r mice at 3 weeks of age is reversed by 6 weeks of age, likely due the engagement of compensatory mechanisms (28).

Previous studies by Bikle et. al. (38) led to a different conclusion regarding the mode of GH action in the skeleton. These authors reported that GH treatment was able to increase bone formation rates in mice globally deficient in IGF-1. In this regard, significant differences in model systems are relevant and may explain the apparent differences in the difference in the conclusions reached by these investigators. Mice globally deficient in IGF-1 exhibit severe disturbances in postnatal growth and sex hormone production. Thus, administration of GH to these animals might have altered bone formation secondary to a correction of growth plate abnormalities or gonadal hormone status. Additionally, because these mice possess intact GHR and IGF-1R, it is possible that these receptors might interact upon GH binding and enable activation of IGF signaling pathways in the absence of IGF-1. Most important, is the possibility that GH stimulated production of IGF-2 which could have compensated for the loss of IGF-1. In our model, re-
moval of the IGF-1R eliminates all IGF-ligand signaling and hence the possibility of compensation by IGF-2.

It is important to emphasize that the conclusions from our studies are relevant to mature osteoblasts and do not rule out possible direct effects of GH which might occur on different populations of skeletal cells or in other GH responsive tissues. In this regard, Wang, et. al. compared long bone growth in mice with targeted deletions of either IGF-1 or GHR (44). Mice lacking IGF-1 with elevated GH had a reduction in chondrocyte hypertrophy whereas in mice lacking GHR, both chondrocyte generation and hypertrophy were compromised, producing a compound deficit in long bone growth. Such observations support dual roles for GH in promoting longitudinal bone growth: an IGF-1-independent role in growth plate chondrocyte generation and an IGF-1-dependent role in promoting chondrocyte hypertrophy. Furthermore, in skeletal muscle, loss of STAT5 (STAT5MKO) causes marked sarcopenia in association with dramatic reductions in muscle IGF-1 production (45). However, recent work by Sotiropoulos et. al. (21) showed that GH signaling profoundly influenced muscle mass, predominantly by promoting fusion of myoblasts with nascent myotubes. In vitro assays demonstrated this GH effect was exerted in an IGF-1-independent fashion, albeit without direct demonstration by blockade of IGF-1 signaling. These data appear to contrast with those obtained in vivo with the STAT5MKO mouse, likely due to significant differences in model systems. The GHR-/- mice used in the studies by Sotiropoulos et. al. have overall growth disturbances and problems with reproductive hormone status that limit interpretation of results from this system. Despite these differences, these studies of GH action in muscle, the growth
plate, and bone provide evidence that the extent to which GH and IGF-1 act independently, in tandem, or even synergistically, likely depends on the tissue and context.

Our studies in mice reported herein have implications for the use of GH in humans. Postmenopausal women treated with rhGH for 36 months showed a 5% increase in total body and 14% increase in lumbar spine bone mineral content after 4 years (46). IGF-1 levels increased from ~150ug/L to ~350ug/L in patients treated with GH. Further, Szulc and colleagues have shown a positive correlation between serum IGF-1 levels and bone mineral density in a cohort of 721 men age 19-85 (47). Such studies in humans suggest that GH increases bone mineral density primarily by elevating IGF-1. This notion is further supported by a case study of a patient with homozygous, partial deletion of the IGF-1 gene (48). Despite abnormally high circulating GH levels, this patient suffered severe osteopenia of the lumbar spine.

In summary, we have established a model system to explore the interactions between GH and IGF-1 signaling in osteoblasts. Using this system, we demonstrate that GH exerts anti-apoptotic actions through IGF-1 independent mechanisms but cannot acutely increase osteoblast numbers in vivo in the absence of an intact IGF-1R, at least under the experimental conditions used here. Thus, we conclude that the main anabolic activity of GH is exerted through increasing the production of IGF-1 and the subsequent action of this growth factor on osteoblast proliferation.
References


Footnotes

We thank the MERCORE laboratory for serum IGF-1 measurements. This work was supported by a VA Merit Review grant and NIH grant R01 AR052746.
Fig. 1. Cre-mediated excision of IGF-1R in primary mouse osteoblasts. Cre excision efficiency was first assessed in the ROSA26 reporter locus. Calvarial osteoblasts were isolated from newborn ROSA26 reporter mice. The cells were infected with AdGFP as control or AdCre for deletion. After two days in culture, cells were stained for LacZ (A). Cre excision efficiency was then assessed in the \(Igf1r\) locus. Calvarial osteoblasts isolated from newborn mice carrying floxed IGF-1R alleles were infected with AdGFP as control or AdCre for deletion. After two days in culture, cells were analyzed for IGF-1R mRNA and protein expression by real-time PCR (B) and immunoblotting (D), respectively. GHR protein level was also analyzed by immunoblotting (C). Control or \(\Delta IGF-1R\) osteoblasts were serum starved for 24 hours before induction of proliferation with PDGF. A portion of cells were lysed 10 minutes following stimulation, and cell lysates were immunoblotted with anti-phospho-ERK (E). The remaining cells were treated with BrdU for the final 12 hours before harvesting at 48 hours of treatment for BrdU and 7-AAD staining. The fold increase of BrdU incorporation following PDGF treatment is shown here for both control and \(\Delta IGF-1R\) osteoblasts (F). Data from three separate experiments are represented as mean ± S.E. *, \(P < 0.05\).
Fig. 2. GHR signaling in osteoblasts lacking the IGF-1R. Control or ΔIGF-1R osteoblasts were grown to confluence and serum starved for 24 hours before stimulation with vehicle (NT), GH or IGF-1 for 10 minutes. Cells were lysed and cell lysates immunoblotted with anti-phospho-STAT5 and anti-STAT5 (A), anti-phospho-Akt and anti-Akt (B), or anti-phospho-ERK and anti-ERK (C). Immunoblots were quantified by densitometry and plotted to compare the ratio of phospho- to total protein in control cells (black bars) and ΔIGF-1R cells (striped bars). Data from three separate experiments are represented as mean ± S.E. *, P < 0.05. Two-way ANOVA showed significant differences among treatment groups in control and ΔIGF-1R for both phospho-ERK and phospho-Akt, (P < 0.05).
Fig. 3. IGF-1R is not required for GH induced IGF-1 and IGFBP-3 mRNA production. Control or ΔIGF-1R osteoblasts were grown to confluence and serum starved for 24 hours before stimulation with GH for 48 hours. Total RNA was extracted and quantitative real-time PCR for IGF-1 in control cells (black bars) and ΔIGF-1R cells (striped bars) (A) and IGFBP3 in control cells (black bars) and ΔIGF-1R cells (striped bars) (B) was performed. Data from three separate experiments are represented as mean ± S.E. *, $P < 0.05$. The difference in fold changes in GH induced IGF-1 and IGFBP3 are not statistically significant between control and ΔIGF-1R osteoblasts ($P > 0.05$).
Fig. 4. GH reduces osteoblast apoptosis regardless of IGF-1R status. Control or ΔIGF-1R osteoblasts were grown to confluence and pre-treated with GH in 1% serum containing media for 24 hours before induction of apoptosis with staurosporine. After 24 hours, a portion of the cells were lysed and lysates immunoblotted with anti-BAX (A). TUNEL staining was also performed (B) and is depicted both by representative fields as well as quantitation from direct counting represented as mean ± S.E. *, P < 0.05.
Fig. 5. GH induced proliferation is ablated upon IGF-1R removal or IGF-1 neutralization. Control or ΔIGF-1R osteoblasts were cultured at low density and starved for 24 hours before stimulation with GH or IGF-1 to induce proliferation. In separate experiments proliferation was assessed by BrdU incorporation in control cells (black bars) and ΔIGF-1R cells (striped bars) (A) or by CellTiter® in control cells (black bars) and ΔIGF-1R cells (striped bars) (B). Wild type osteoblasts were serum starved and treated with GH or IGF-1 in the presence of an IGF-1 neutralizing antibody or IgG control before assessment of proliferation with CellTiter® (C). Data from three separate experiments are represented as mean ± S.E. *, P < 0.05. Two-way ANOVA for the neutralizing antibody studies (C) showed significant differences among GH and IGF-1 treatment groups (P < 0.05) in IgG and anti-IGF-1 neutralizing antibody groups.
Fig. 6. GH requires the IGF-1R to induce osteoblast proliferation in vivo. Control (Igf1r^{flox/flox}) and ΔIgf1r mice (Igf1r^{flox/flox Cre+}) 3 weeks of age were treated subcutaneously with GH or vehicle for 7 consecutive days. Mice were then sacrificed and distal femora sectioned and analyzed histomorphometrically. Representative histological sections from control vehicle treated (a), control GH treated (b), ΔIgf1r vehicle treated (c), and ΔIgf1r GH treated (d) are shown here. Serum samples from mice were analyzed for IGF-1 levels (e). Relevant histomorphometric parameters of osteoblast number per trabecular area (f) and bone formation rate (g) are also illustrated here. *, P < 0.05
OSTEOBLAST-SPECIFIC ABLATION OF GROWTH HORMONE RECEPTOR
RESULTS IN COMPARTMENT AND GENDER-SPECIFIC BONE LOSS

by

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Abstract

Growth hormone (GH) exerts profound anabolic actions during postnatal skeletal development, in part, through stimulating the production of insulin-like growth factor-1 (IGF-1) in liver and bone. We previously reported that the ability of GH to induce osteoblast proliferation depended on the IGF-1 receptor (IGF-1R). In this study, we used a Cre-loxP recombination strategy to specifically delete GHR from osteoblasts and further investigate the roles of GH and IGF-1 in osteoblast function \textit{in vitro} and \textit{in vivo}. Primary calvarial osteoblasts from mice carrying floxed GHR alleles (GHR\textsuperscript{flx/flx}) infected with adenoviral vectors expressing the Cre recombinase (AdCre) demonstrated marked reductions in GHR mRNA (80-90\%) and no detectable GHR protein as assessed by immunoblotting. As expected, GHR disruption abolished GH induced JAK2/STAT5 phosphorylation. IGF-1 induced ERK phosphorylation was blunted in ΔGHR osteoblasts, while Akt phosphorylation was only modestly decreased. BrdU incorporation increased in response to GH and IGF-1. GHR disruption eliminated GH-induced BrdU incorporation, and blunted IGF-1 induced BrdU incorporation. GHR disruption also abolished the anti-apoptotic activity of IGF-1 seen in control osteoblasts. Despite these functional deficiencies, ΔGHR osteoblasts maintained differentiation capability \textit{in vitro} as indicated by alkaline phosphatase staining. Osteoblast-specific disruption \textit{in vivo} resulted in a gender-specific loss of cortical thickness in female 6 week old mice as indexed by μCT. Immunohistochemical staining of femurs from normal mice with antibodies specific for GHR revealed the highest GHR immunoreactivity at the periosteal surface of the cortex. Histomorphometric analysis of female 6 week old mice showed a marked increase in osteoblast number but impaired mineralization capability as indicated by increases in os-
teoid volume, osteoid surface, and mineralization lag time. These results suggest that GHR is required for the full action of IGF-1 in osteoblasts \textit{in vitro}, but this deficit can be partially compensated \textit{in vivo} except in areas of the highest GHR concentration, e.g. cortex.
Introduction

Osteogenesis and proper skeletal remodeling depend on the coordinated activities of three principle cell types: osteoblasts, osteoclasts, and osteocytes. Osteoblast activity is regulated by a number of local growth factors, cytokines, and systemic hormones (1,2). Growth hormone (GH) and insulin-like growth factor-1 (IGF-1) are two key components of this anabolic milieu. GH is a cytokine peptide (3) produced and stored by somatotroph cells of the anterior pituitary. GH functions by binding to its homodimeric transmembrane GH receptor (GHR), triggering conformational changes (4) that lead to activation of associated JAKs (5-7) to activate STATs (8-16), PI3K/Akt (17,18), and ERK (19-21). GHR is devoid of any intrinsic kinase activity, with activation of downstream signaling resulting from the kinase activity of associated JAK2. Additionally, JAK2 has recently been described to play a role in processing and stabilization of GHR (22). Of note, while STAT5b induced GH target gene transcription (most notably, IGF-1) requires tyrosine phosphorylation of GHR cytoplasmic domain, ERK activation requires only JAK2 activation (20,23-25).

GH actions are mediated predominantly by its stimulation of the production of IGF-1 from liver and peripheral tissues. IGF-1 is a widely expressed polypeptide that bears homology to pro-insulin. IGF-1 signals primarily via the heterotetrameric type-1 IGF-1 receptor (IGF-1R) to trigger ERK and PI3K/Akt activation through SHC and insulin receptor substrates-1 and 2 (IRS-1/2) (26-27). The effects of IGF-1 in bone are well characterized. IGF-1 is an important survival factor for many cell types, including osteoblasts (28), and induces proliferation of MC3T3 osteoblast-like cells (29). IGF-1 also plays a role in differentiation of fetal rat calvarial osteoblasts to augment type I collagen
synthesis and inhibit collagen degradation (30). Osteoblast-specific overexpression of IGF-1 in mice accelerates new bone formation as well as increases the rate at which matrix is mineralized (31), while osteoblast-specific disruption of the IGF-1R causes markedly impaired mineral apposition rate and increased mineralization lag time (32).

Because GH stimulates IGF-1 production in many tissues, it has been difficult to define specific effects of GH in bone. High affinity GH receptors are expressed by rat osteoblast-like UMR106 osteosarcoma cells (33) and human (34) and mouse (35-37) primary cultured osteoblasts. GH induces osteoblast proliferation in cell culture (34), but until recently, the relative contribution of GH and IGF-1 to this effect could not be determined. Mice globally deficient in IGF-1 or GH provided indirect evidence for independent functions of GH and IGF-1 on osteoblast mediated bone formation. While both of these mutant animals had markedly impaired postnatal BMD, the defect was more severe in IGF-1 nulls, as reported by Mohan and colleagues (38). Interpretation of these models is confounded, however, by severe disturbances of overall growth of these mice and problems with reproductive hormone status.

Recently, our group employed a Cre/LoxP method to disrupt IGF-1R specifically in osteoblasts, eliminating the confounding effects of GH induced IGF-1 and facilitating study of GH actions in osteoblasts (39). We now use a Cre/LoxP method to selectively disrupt GHR in osteoblasts both in vitro and in vivo and further examine GH/IGF-1 action in bone. Our results suggest that GHR is required for the full action of IGF-1 in osteoblasts in vitro, but this deficit can be partially compensated in vivo except in areas of the highest GHR concentration, e.g. cortex.
Experimental Procedures

Materials

Cell culture media, α-minimal essential medium (αMEM), was obtained from Cellgro-Mediatech (Herdon, VA) and fetal bovine serum (FBS) was from Gibco (Gaithersburg, MD). Bovine GH was obtained from the National Hormone and Peptide Program – Monsanto (Torrance, CA) and stored in 200X aliquots for single use. Human IGF-1 was obtained from GroPep (Theberton, SA, Australia) and stored in 1000X aliquots for single use. Antibodies used for immunoblotting included anti-β-actin (C4) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-phospho-JAK2 (Tyr1007/1008), anti-JAK2, anti-phospho-STAT5 (Tyr 694), anti-STAT5, anti-phospho-Akt (Ser 473), anti-Akt, anti-phospho-ERK (Thr202/Tyr204), anti-ERK from Cell Signaling Technology (Danvers, MA); anti-GHR (AL47) was a generous gift from Dr. Stuart Frank. Horseradish-peroxidase conjugated rabbit and mouse secondary antibodies were obtained from Pierce Biotechnology (Rockford, IL). Polyvinylidene difluoride (PVDF) membrane, Laemmli sample buffer and other electrophoresis supplies were from Bio-Rad. Assay kits for flow cytometry analysis of cell proliferation and apoptosis were purchased from BD Pharmingen (San Jose, CA). All other reagents not specified here were purchased from Sigma.

Immunohistochemistry

IGF-1R IHC: After standard rehydration procedures (in xylene and ethanol), paraffin sections were treated with 0.1% TX-100 in PBS for 10 min. followed by antigen retrieval by boiling slides/sections in 1mM EDTA (pH 8.0) for 10-15 min. Slides were
then washed with PBS and treated with 3% H₂O₂ for 10 min. Sections were blocked with Avidin and Biotin (Vector Labs Inc, Cat# SP-2001) according to manufacturer’s instructions and then treated with 2% normal goat serum in 1% BSA at room temperature for 20 min. Sections were washed with PBS and stained with IGF-1Rβ rabbit polyclonal primary antibody (Cell signaling; Cat# 3027) at 1:200 dilution in 1% BSA overnight at 4°C. Slides were washed with PBS and stained with goat anti-rabbit secondary antibody for 1 hour at room temperature. Slides were then treated with ABC reagent (R.T.U Vectastain; Vector Labs Inc, PK-7100) at RT for 30 min followed by PBS wash and DAB development for approximately 8 min. Slides were washed in water, dehydrated and mounted with cover slips. Immunohistochemistry for GHR was performed by the same protocol as IGF-1R except antigen retrieval was performed using Pepsin solution (Digest-All 3; Zymed, Cat# 00-3009) at 37°C for 15 min and primary antibody was anti-GHR (AL47).

**Osteoblast isolation and culture**

Osteoblasts were isolated from calvaria of newborn wild-type or Ghr<sup>flx/flx</sup> mice by serial digestion in 1.8 mg/ml collagenase type I (Worthington, Lakewood, NJ) solution. Calvaria were digested in 10 ml of digestion solution for 15 min at 37 °C with constant agitation. The digestion solution was collected, and digestion was repeated with fresh digestion solution an additional four times. Digestions 3–5 (containing the osteoblasts) were pooled together, centrifuged, washed with αMEM containing 10% FBS, 1% pen/strep, and plated overnight at 37 °C in a humidified incubator supplied with 5% CO₂. For in vitro deletion of the GHR, osteoblasts containing floxed GHR alleles were cultured to be 70% confluent and then, in the absence of serum, were infected with ade-
novirus encoding Cre recombinase (Ad-Cre) (Vector Biolabs, Philadelphia, PA) at a titer of 100 multiplicity of infection (moi). Infection with 100 moi of adenovirus encoding green fluorescent protein (Ad-GFP) (Vector Biolabs) was used as control. After 1 hr, culture medium containing 10% FBS was added and the cells were allowed to recover for the next 48 hr. Greater than 80% GHR deletion was confirmed for every infection by quantitative real-time PCR and immunoblotting.

*Cell lysis and immunoblotting analysis*

For signaling experiments, osteoblasts were cultured in 10% FBS αMEM to 90% confluence and then serum starved in 0.1% FBS containing αMEM for 24 hr to reduce cellular activity to quiescent levels prior to stimulation. At the end of the study, the cells were washed twice with ice cold phosphate-buffered saline (PBS) and resuspended in lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM MgCl₂, 1 mM EDTA, 1% Triton X-100, and 10% glycerol). Protease and phosphatase inhibitors (Sigma) were added to the lysis buffer. The cell lysates were homogenized by needle aspiration and protein concentration was measured by Bradford protein assay (Bio-Rad). For immunoblotting of whole cell lysates, equal amounts of protein (10 or 20 µg/lane) were solubilized in Laemmli sample buffer and loaded onto a mini-SDS-PAGE system. Following electrophoresis, proteins were transferred to a PVDF membrane using a Bio-Rad semi-dry transfer system. Protein transfer efficiency was verified using prestained protein markers. Membranes were then blocked with 5% non-fat dry milk for 1 hr at room temperature and subsequently incubated overnight at 4°C with antibodies directed against the protein of interest. Signals were detected using a horseradish peroxidase-conjugated secondary
antibody and bound antibodies were visualized using the Supersignal West Femto Substrate (Pierce). Western blot photographic results were scanned with a Canon flatbed scanner.

Quantitative real-time PCR

Total RNA was extracted from cells using the TRizol® method as recommended by the manufacturer (Invitrogen). The RNA concentration was estimated spectrophotometrically and only pure RNA (A260:A280 ratio ≥ 1.8) was used for further analysis. First strand cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad). The cDNA was amplified in the Opticon Continuous Fluorescent Detector (MJ Research, Waltham, MA) using IQ™ SYBR Green supermix (Bio-Rad) and sequence specific primers. PCR reactions were performed in triplicate for each cDNA, averaged, and normalized to endogenous β-actin reference transcripts. Primer sequences used were as follows: GHR: F5’-GATTTTACCCCCAGTCCCAGTT-3’, R5’-GACCCTTCAGTCTTCTCATCCACA-3’; IGF-1: F5’-GCTCTTCAGTTCGTGTGTGGAC-3’, R5’-TTGGGCTGTCAGTGTGGCGC-3’; β-actin: F5’-ACCTCCTACAATGAGCTGC-3’, R5’-TGCCAATAGTGATGACCT-3’.

Osteoblast proliferation assays

Control and ΔGHR osteoblasts were plated in 6-well plates at low cell density (9 X 10⁴ cells per well) and cultured in αMEM with 1% FBS for 24 hours to arrest the cells in G0 phase. The cells were then restimulated with 500ng/mL GH or 100ng/mL IGF-1 for 48 hours. For proliferation analysis of the cells, 10 μM BrdU was added to the me-
dium at the time of mitogenic stimulation. The cells were then stained with anti-BrdU-APC and 7-AAD and analyzed by FACS Calibur (Becton-Dickson). 20,000 events were collected for each sample and results were analyzed with WinMDI version 2.8.

Osteoblast apoptosis assays

Control and ΔGHR osteoblasts were plated at confluence in 96 well plates in 1% FBS containing media. They were then pretreated for 24 hours with GH or IGF-1 before induction of apoptosis (8ng/mL staurosporine). Apoptosis was assessed by Promega CaspaseGlo 3/7 according to manufacturer’s instructions after 8 hours.

Animal studies

Female control (GHR^{flox/flox}) and ΔGHR mice (GHR^{flox/flox Cre+}) were sacrificed at 6 weeks of age and femurs were removed and fixed in 100% ethanol. Micro-computed tomography analysis (µCT) of the femur was performed at the Orthopedic Biomechanics Laboratory at Beth Israel Deaconess Medical Center (Boston, MA). Histological analysis was performed at the Albert B. Chandler Medical Center at the University of Kentucky (Lexington, KY).

Statistical Analysis

All statistical analyses were performed using the Microsoft Excel data analysis program for ANOVA or Student’s t-test analysis with an assigned significance level of 0.05 (α). All experiments were repeated at least three times unless otherwise stated. Values were expressed as a mean ± S.E.
Results

Localization of GHR in mouse bone

As a first step in studying GHR function in osteoblasts, we determined the pattern of expression of each receptor in bone from normal mice using immunohistochemistry with antibodies specific for each receptor. GHR immunoreactivity was observed in osteoblasts and osteocytes in both cortical (Fig. 1A) and trabecular bone (data not shown). Interestingly, osteoblasts on the surface of the cortex appeared to exhibit greater immunoreactivity compared with osteoblasts in trabecular bone (Fig. 1A, arrows). IGF-1R immunoreactivity was more evenly distributed in all osteoblasts and osteocytes (Fig. 1B).

Disruption of GHR in osteoblasts in vitro

The effect of loss of GHR signaling was initially examined in primary osteoblasts in vitro. Primary osteoblasts from mice homozygous for the GHR floxed alleles were infected with Cre expressing adenovirus constructs (Fig. 2A, top). Real time PCR analysis of GHR mRNA expression (Fig. 2B) indicated ~90% deletion of GHR mRNA at a 100 moi dose of AdCre and complete loss of GHR protein (Fig. 2C). Importantly, IGF-1R protein levels were unaffected following disruption of GHR (Fig. 2C).

We next determined the effect of GHR deletion on GH and IGF-1 generated signals. As shown in Figure 3A, infection of wild-type osteoblasts with AdCre had no discernable effects on basal or GH and IGF-1 induced ERK phosphorylation at 10 minutes. This result ensured changes in GH/IGF-1 signaling were the direct consequence of GHR loss. Osteoblasts from mice carrying floxed Ghr alleles were infected with AdGFP (hereafter referred to as control) or AdCre (hereafter referred to as ∆GHR) and treated
with GH or IGF-1 for 10 minutes. As expected, disruption of GHR abolished GH stimulated JAK2 (Fig. 3B – upper bands) and STAT5 (Fig. 3C) phosphorylation. IGF-1 does not activate this pathway. In addition, GH induced ERK phosphorylation was eliminated by GHR disruption (Fig. 3D). IGF-1 induced ERK activation was also significantly blunted following GHR removal (Fig. 3D). By contrast, disruption of GHR resulted in only a modest reduction in IGF-1 induced Akt phosphorylation (Fig. 3E). These results suggest that IGF-1 requires GHR to fully activate downstream signaling, specifically, ERK activation.

Removal of GHR dampens osteoblast proliferation in response to IGF-1

We next determined the effect of GHR disruption on osteoblast proliferation. Control and ΔGHR osteoblasts were serum starved for 24 hours and then treated with vehicle, GH or IGF-1. Proliferation was assessed after 48 hours by flow cytometry for BrdU incorporation. In control osteoblasts, GH and IGF-1 significantly induced osteoblast proliferation as measured by BrdU uptake (Fig. 4A). Disruption of GHR abolished GH induced BrdU uptake (Fig. 4A). However, IGF-1 significantly induced BrdU uptake in ΔGHR cells, although the magnitude of this effect was blunted (Fig. 4A).

IGF-1’s anti-apoptotic activity is abolished by disruption of GHR

To determine the requirement of GHR for the anti-apoptotic activity of IGF-1, control and ΔGHR osteoblasts were pretreated with vehicle, GH or IGF-1 in 1% serum containing media before the induction of apoptosis with staurosporine. Apoptosis was measured by Promega Caspase-Glo™ 3/7 assay. In control osteoblasts, GH and IGF-1
significantly reduced apoptosis (Fig. 4B). As expected, disruption of GHR abolished the anti-apoptotic activity of GH (Fig. 4B). In addition, the ability of IGF-1 to blunt staurosporine induced apoptosis was also lost in ΔGHR osteoblasts (Fig. 4B).

*Lack of effect of GHR disruption on osteoblast differentiation*

Our previous studies have shown that IGF-1 enhances osteoblast differentiation as indexed by alkaline phosphatase staining. To assess the effect of GHR signaling on osteoblast differentiation *in vitro*, control and ΔGHR osteoblasts were grown to confluence and then switched to differentiation media supplemented with vehicle, GH or IGF-1 for 10 days. Differentiation was then assessed by ALP staining (Fig. 5). Disruption of GHR had no apparent effect on osteoblast differentiation.

*Osteoblast-specific ablation of GHR in vivo results in a gender-specific cortical thinning*

To now determine the effect of GHR disruption on osteoblasts *in vivo*, μCT analysis was performed on the femurs of 6 week old male and female control (GHR<sup>flox/flox</sup>) and ΔGHR mice (GHR<sup>flox/flox Cre+</sup>). Measurements of trabecular bone remained unchanged in female ΔGHR mice (Fig. 6A & 6C), while mid-femoral cortical measurements showed a significant decrease in cortical thickness (Fig. 6B & 6C). Cortical bone area was also decreased although this effect did not reach statistical significance (data not shown). No significant differences were seen in any parameters for male 6 week old ΔGHR mice (Fig. 6A & 6B).
Histomorphometric analysis of ∆GHR mice

To further assess the effect of GHR deletion on bone volume \textit{in vivo}, histomorphometric analysis was performed at the distal femur of 6 week old male and female control and ∆GHR mice. Female ∆GHR mice had increased osteoblast numbers (Fig. 7A), but these osteoblasts had a mineralization defect defined by increased osteoid volume (Fig. 7B) and mineralization lag time (Fig. 7C). Male mice displayed a similar, although less pronounced, phenotype (Fig. 7A-C). While there were striking similarities to the phenotype of osteoblast-specific IGF-1R knockout mice (32), a number of important differences should be noted. Mice lacking GHR showed no difference in trabecular bone volume, thickness, number, or separation by µCT or histomorphometric analysis (all decreased in ∆IGF-1R mice).
Discussion

In these studies, we used a Cre-LoxP technique to selectively disrupt the GHR in osteoblasts *in vitro* and *in vivo* and further examine GH/IGF-1 action in bone. Importantly for our studies, IGF-1R remained unchanged following GHR disruption. In control osteoblasts, GH and IGF-1 induced ERK phosphorylation. Upon disruption of GHR, there was a slight increase in basal ERK phosphorylation, and GH effects were abolished. Indeed, we did find a reduction in IGF-1 induced ERK phosphorylation in ΔGHR osteoblasts to suggest that GHR facilitates maximal IGF-1 signaling. Akt phosphorylation, in contrast to ERK, was only modestly reduced by GHR disruption. Studies by Huang et al. (40) describing a physical interaction of GHR, JAK2, and IGF-1R may provide an explanation for the differential loss of IGF-1 induced ERK versus Akt phosphorylation in ΔGHR osteoblasts. GHR and IGF-1R are both known to activate MAPK signaling pathways in a SHC-Grb2-SOS-Ras-Raf dependent manner, and JAK2 binds and phosphorylates SHC to activate this pathway downstream of GHR (41). In this manner, GHR disruption may impair IGF-1 induced recruitment and/or phosphorylation of SHC-Grb2-SOS through IGF-1R. IGF-1 induced Akt phosphorylation results from IRS-1/2 phosphorylation and activation of the PI-3K pathway (42). IRS proteins are intimately associated with IGF-1R (42-44) and may be unaffected by disruption of GHR and loss of the aforementioned GHR/JAK2/IGF-1R complex. Further examination of the molecular mechanisms responsible for GHR/IGF-1R collaboration in osteoblasts is currently underway in our laboratory.

Regardless of the exact mechanism responsible, the deficits in osteoblast signaling following GHR disruption were also visible in assays of osteoblast proliferation and
apoptosis. GHR disruption eliminated GH induced proliferation and blunted osteoblast responsiveness to IGF-1, in accord with the signaling studies. Disruption of GHR also abolished the anti-apoptotic activity of IGF-1. Loss of anti-apoptotic activity of IGF-1 despite only a modest reduction in Akt suggests that IGF-1 exerts this action in osteoblasts primarily through some other pathway. Recent studies support the anti-apoptotic role of the MAPK pathway in osteoblasts. Liang et. al. (45) show increased ERK activation in osteoblasts following serum deprivation and activation of the intrinsic death pathway. Blocking ERK activation with a pharmacological inhibitor, U0126, markedly increased apoptosis in their system.

Micro-CT analysis of bones from mice lacking GHR specifically in osteoblasts revealed a reduction in cortical bone volume that was restricted to females. Histomorphometric analysis of these bones also showed decreased cortical bone volume, although it did not reach statistical significance by this measure. Histomorphometric analysis revealed a more striking phenotype in the trabeculae however, that in some aspects closely resembled the phenotype of mice with osteoblast-specific disruption of IGF-1R. Namely, these mice had increased osteoblast numbers with increased osteoid volume and increased mineralization lag time. This result suggests a few important points concerning GH/IGF-1 action in bone. First, immunohistochemical staining with antibodies specific for GHR indicated greater GHR immunoreactivity along the periosteal surface of the cortex (Fig. 1A). Combined with the results of our previous studies showing that the proliferative effects of GH on osteoblasts in vivo required the IGF-1R (39), it can be inferred that GHR serves primarily as a spatial and compartmental regulator of IGF-1 levels within bone. It is possible that in the case of our model system, the deficit in osteoblast
activity following GHR removal, and subsequent decrease in local IGF-1, was com-
pounded because of high GHR concentration in the cortex. Thus, the phenotype was
more dramatic in the cortex than the trabeculae, where a number of other IGF-1 produc-
ing cells in the local stroma and marrow might help to compensate this loss. Second, the
cortical phenotype was seen only in females, and the histomorphometric alterations were
more dramatic in females as well. This supports the notion that sex-dependent differ-
ences in temporal pulsatility of GH from the pituitary, as well as the sexual dimorphism
of GH target genes and its interaction with sex steroids, especially estrogen (46), play an
important role in GH action in bone. It was previously accepted that androgens stimu-
lated periosteal expansion, whereas estrogens decreased periosteal bone formation (47).
It has since been shown, in human males with aromatase deficiency, that low levels of
estrogen are required for periosteal expansion (48). This biphasic role of estrogen at the
periosteum, where higher levels seen in females inhibit periosteal bone formation, could
compound the effect of GHR loss in our model system and explain the gender specificity
of the cortical phenotype.

In summary, we demonstrate that GHR is required for a maximal osteoblast re-
response to IGF-1. Taken together with previous studies of IGF-1R in osteoblasts, we con-
clude that GHR and IGF-1R must both be present to fully activate either signaling path-
way. Further, while GHR might be required to fully activate IGF-1R signaling in vitro,
our results from ΔGHR mice would suggest that this deficit can be compensated in vivo
except in areas of highest GH receptor concentration, e.g. cortex.
References


Footnotes

This work was supported by a VA Merit Review grant and NIH grant R01 AR052746.
Fig. 1. Immunohistochemical staining of GHR and IGF-1R in 6 week old mouse femur. Paraffin embedded sections from 6 week old female mice were stained with anti-GHR (A), anti-IGF-1R (B) or non-immune serum (C) and developed with DAB. Images shown are 40X magnification. Arrows indicate greater GHR immunoreactivity along the periosteal surface of the cortex.
Fig. 2. Cre-mediated excision of GHR in primary mouse osteoblasts. (A) Cre-loxP gene deletion strategy. (B) Calvarial osteoblasts isolated from newborn mice carrying floxed GHR alleles were infected with AdGFP as control or varying doses of AdCre for deletion. After two days in culture, cells were analyzed for GHR mRNA by real-time PCR, and 100 moi dose was selected for all further experiments. (C) Protein levels of GHR and IGF-1R were then analyzed by immunoblotting.
**Fig. 3.** IGF-1 induced ERK and Akt phosphorylation is blunted in ΔGHR osteoblasts. Control or ΔGHR osteoblasts were grown to confluence and serum starved for 24 hours before stimulation with vehicle, GH or IGF-1 for 10 minutes. Cells were lysed and cell lysates immunoblotted with (B) anti-phospho-JAK2 (upper band) and anti-JAK2, (C) anti-phospho-STAT5 and anti-STAT5, (D) anti-phospho-ERK and anti-ERK, or (E) anti-phospho-Akt and anti-Akt. Non-floxed, wild-type cells were infected and treated identically and served as control for effects of viral infection on ERK signaling (A). Blots are representative of at least 3 separate experiments.
Fig. 4. Proliferative and anti-apoptotic actions of IGF-1 are blunted in ΔGHR osteoblasts. (A) Control or ΔGHR osteoblasts were cultured at low density and starved for 24 hours before stimulation with GH or IGF-1 to induce proliferation. Proliferation was assessed by BrdU incorporation. (B) Control or ΔGHR osteoblasts were plated at confluence in 1% FBS containing media and pretreated for 24 hours with GH or IGF-1 before induction of apoptosis (8ng/mL staurosporine). Apoptosis was assessed by Promega CaspaseGlo 3/7 after 8 hours. *, $P < 0.05$. 
Fig. 5. Alkaline phosphatase staining is unaffected by GHR disruption. Control or ΔGHR osteoblasts were grown to confluence and then switched to differentiation media (normal growth media with β-glycerophosphate and ascorbic acid) supplemented with GH or IGF-1. Medium was changed every other day for a total of 10 days before alkaline phosphatase (ALP) staining.
Fig. 6. Osteoblast-specific ablation of GHR in vivo results in a gender specific cortical thinning. Femurs of 6 week old female ΔGHR or control littermates were examined by μCT. Trabecular parameters remained unchanged (A and B), while cortical thickness was significantly decreased in ΔGHR animals (C) and there was a trend toward decreased cortical bone area (D). Representative μCT images are shown in E. *, P < 0.05.
Fig. 7. Histomorphometric analysis of ΔGHR mice. Histomorphometric measurements were made on the distal femur of 6 week old female control (n=6) and ΔGHR (n=5) mice. (A) Number of osteoblasts per bone perimeter, (B) osteoid volume per bone volume, and (C) mineralization lag time *p<0.05.
CONCLUSIONS AND FUTURE DIRECTIONS

In this dissertation project, I have presented genetic evidence which suggests the main anabolic action of GH in bone is exerted by its ability to stimulate the local production of IGF-1. Removal of IGF-1R from osteoblasts resulted in sub-maximal GH signaling in vitro and a complete ablation of the anabolic effects of GH treatment on osteoblast proliferation normally seen in vivo. Conversely, removal of GHR from osteoblasts blunted IGF-1 signaling in vitro, indicating a reciprocal relationship between these receptors to facilitate maximal activation of either pathway. Mice lacking GHR in osteoblasts displayed similarities to the phenotype of osteoblast-specific IGF-1R deficient mice with certain notable differences, e.g. no change in trabecular bone volume and female-specific cortical thinning.

As described in the Introduction, the complex interconnectedness of the GH/IGF-1 axis has proved to be a significant hurdle in fully dissecting the individual contributions of GH and IGF-1 to bone anabolism. Previous genetic models of global GHR and IGF-1 deletion (38) provided initial insights into GH and IGF-1 action in bone, but results from these model systems were confounded by growth disturbances in other organ systems and problems with reproductive hormone status. Employing sophisticated genetic models of tissue-specific gene deletion through a Cre-loxP recombination strategy, we first specifically disrupted IGF-1R in osteoblasts. This allowed examination of GH action without the confounding effects of subsequent IGF-1R activation and signaling. The results of
these studies showed that the proliferative effects of GH in osteoblasts, both \textit{in vitro} and \textit{in vivo}, required the presence of an intact IGF-1R. We also noted diminished JAK/STAT activation in osteoblasts lacking IGF-1R, a finding that suggested both receptors are required for maximal activation of GH signaling.

We next performed the converse experiments, using the same Cre-loxP technique to selectively remove GHR from osteoblasts. Disruption of GHR in osteoblasts blunted IGF-1 signaling, indicating a reciprocal relationship for the requirement of both receptors to elicit a maximal downstream signal from either ligand. Functional assessment of \(\Delta\)GHR osteoblasts \textit{in vitro} revealed a diminished proliferative and anti-apoptotic response to IGF-1, but no effect on differentiation. \textit{In vivo}, osteoblast-specific GHR disruption resulted in female-specific cortical thinning as indexed by \(\mu\)CT. Histomorphometric analysis of the trabecular compartment displayed certain phenotypic characteristics that resembled \(\Delta\)IGF-1R mice, namely increased osteoblast numbers and unmineralized osteoid. By contrast, \(\Delta\)GHR mice had no decrease in trabecular bone volume, trabecular number, thickness, or separation seen in \(\Delta\)IGF-1R mice.

A comparison \(\Delta\)IGF-1R mice and \(\Delta\)GHR mice described in this dissertation project would suggest a compartment-specific model for GH action in osteoblasts (Fig. 1), wherein GH, acting through its receptor, primarily serves to augment local IGF-1 production. This IGF-1 is required for osteoblasts to mineralize newly formed matrix, both \textit{in vitro} (72) and \textit{in vivo} (58). The deficits in osteoblast signaling and function, particularly proliferation, seen upon removal of either IGF-1R or GHR \textit{in vitro} are likely sensed at an early age in the mouse (\(\Delta\)IGF-1R mice have \(\sim\)50\% reduction in osteoblast numbers at 3 weeks of age (58)).
Fig. 1. Proposed model for the compartment-specific effects of GH in bone. (A) In control mice, GH augments local IGF-1 levels in both trabecular and cortical compartments. (B) Upon removal of GHR, osteoblasts along the cortex are limited to IGF-1 and other growth factors from the circulation alone, resulting in reduced bone volume. Osteoblasts in the trabecular compartment, in addition to circulating sources, also maintain IGF-1 and other growth factor stimulus from the surrounding marrow component and are able to achieve normal bone volume.
Upon initiation of pubertal growth, a compensatory burst of as yet undefined mitogenic factors are produced in the bone, likely from various cell types within the marrow compartment. These alternative mitogens then rescue the depleted osteoblast population. However, ΔIGF-1R mice remain deficient in their mineralization capacity and display reduced trabecular bone volume. By contrast, the ability to mineralize in response to IGF-1 remains intact in ΔGHR mice. This rescued population of osteoblasts in the trabecular compartment responds to alternative sources of IGF-1, either from the circulation or other cells within the marrow space, and forms bone normally. Thus, GHR does not appear to be required for formation of trabecular bone.

By contrast, the formation of cortical bone does appear to be dependent on the action of GHR. Osteoblasts in the cortex are not surrounded by marrow cells capable of producing compensatory factors to supplant GH-induced IGF-1 production lost upon GHR removal. Thus, these osteoblasts would be significantly more dependent upon, and sensitive to, loss of autocrine GH-induced IGF-1. This notion is supported by the higher GHR immunoreactivity in the periosteal surface of the cortex as indexed by immunohistochemical staining (Fig. 1A). Osteoblasts on this surface would still have access to liver-derived IGF-1 in the circulation, which might explain why loss of local, osteoblast-produced IGF-1 doesn’t result in more dramatic reduction of cortical bone volume. Studies currently underway to examine the exact contribution of this circulating IGF-1 in bone will be discussed later in this section.

Further characterization of the bone phenotype by µCT and histomorphometry at 3 weeks and 16 weeks of age in the ΔGHR mice should provide additional insight into the temporal changes resulting from loss of GHR throughout and following pubertal
growth. We predict that ΔGHR mice will have decreased osteoblast numbers and bone volume (trabecular and cortical) at 3 weeks, similar to ΔIGF-1R. If the compensatory signals resulting from loss of local IGF-1 production persist after the pubertal growth spurt, we would expect ΔGHR mice to continue to have increased osteoblast numbers with no change or possibly even increased trabecular bone volume, while the cortical phenotype seen in females would persist.

The compartment-specific defect seen in ΔGHR mice might also be explained by fundamental cellular differences between periosteal and trabecular osteoblasts. Recent studies have shown periosteal osteoblasts may respond to PTH differently than osteoblasts found in trabecular bone (73). The potential for a similar mechanism in response to GH will be tested by comparing the cellular signaling, e.g. JAK/STAT phosphorylation, of periosteal-derived osteoblasts versus calvaria-derived osteoblasts. Significantly greater activation or activation of alternative signaling pathways in periosteal osteoblasts would support this mechanism for compartmental specificity of GH in bone.

As mentioned above, this cortical phenotype is restricted to female mice, highlighting the interplay of sex steroids with the GH/IGF-1 axis. The paradigm of androgen/estrogen action in bone was widely accepted to follow that androgens increased periosteal expansion, while estrogens inhibited this process – thus the sexually dimorphic patterns of bone growth in males and females (74). The discovery of human males with aromatase deficiency, thus a complete lack of estrogen, challenged this paradigm. These men had a failure of pubertal periosteal expansion that was corrected with estrogen treatment (75). This clearly demonstrated a role for estrogen in periosteal expansion and suggested a biphasic effect for estrogen, where low levels of estrogen in males had posi-
tive effects and the higher levels seen in females were inhibitory. This model of estrogen interaction at the periosteum presents two possibilities to explain the gender specificity seen in ΔGHR mice. The first possibility is that the inhibitory effects of high estrogen levels at the periosteum compound the effects of loss of local IGF-1 production following GHR removal, thus unmasking the phenotype specifically in females. A second possible explanation would suggest the opposite: loss of local IGF-1 in the cortex is alone sufficient to produce the cortical thinning phenotype, but low levels of estrogen in males actually compensate to mask the phenotype. Studies by Venken et. al. (76) would seem to support this concept, as administration of estrogen to orchidectomized male mice was shown to increase periosteal bone mass in the absence of GHR.

The possibility also exists that estrogen may interfere directly with GH and/or IGF-1 signaling in addition to acting through its own receptors to exert effects. Recent studies by Ogita et. al. have shown that estrogen can interfere with PTH signaling in periosteal osteoblasts (73). This potential mechanism of gender specificity will be tested by treating periosteal-derived osteoblasts with GH alone or a combination of GH and varying doses of estrogen. Examination of GH-induced cell signaling, proliferation, and differentiation in these groups should be revealing with respect to the mode of estrogen action in our ΔGHR model system. If estrogen is inhibiting periosteal expansion at high levels in the female, then higher doses of estrogen should block GH induced proliferation and differentiation. If lower levels of estrogen in the males are stimulating periosteal expansion, we would expect to see no change or perhaps slight increases in GH proliferation and increased differentiation in those groups treated with low estrogen concentrations.
While the studies described above using osteoblast-specific ablation of IGF-1R and GHR have provided considerable insight into the mode of action of GH and IGF-1 in bone, they do not completely address the GH/IGF-1 axis in this regard. Liver-derived circulating IGF-1 can also play a role in bone development. Despite studies in a variety of mouse models – Liver IGF-1 Deficient (LID) mice (32), Acid- Labile Subunit deficient (ALSKO) mice, and LID+ALSKO double knockout (34) – the contribution of circulating and local IGF-1 in dictating normal bone growth and volume remains in question. The models cited above suffer a variety of caveats which prevent clear interpretation of the effect of circulating IGF-1 in bone. Serum IGF-1 in LID mice is reduced by only ~75% with no effect on growth. LID+ALSKO double knockout achieves ~90% reduction in circulating IGF-1 and displays growth retardation, but ALSKO mice alone have a bone phenotype that is likely not related to reduced IGF-1 levels (34).

To test the role of liver-derived IGF-1 on mouse growth, we again employed a Cre-loxP recombination strategy to disrupt liver-derived circulating IGF-1 by specifically deleting GHR from liver (Growth Hormone Receptor Liver Deficient – GHRLD) using Cre recombinase driven by an albumin promoter (Fig. 2A). These studies were performed in collaboration with Dr. Mark Sperling at the University of Pittsburgh and are being prepared for submission. Confirmation of GHR deletion from the liver of 8 week old GHRLD mice was assessed by RT-PCR (Fig. 2B), real-time PCR (Fig. 2C), and immunofluorescent staining with antibodies specific for GHR (Fig. 2D). Serum measurements from 8 week old male and female mice confirmed that circulating IGF-1 was reduced by ~95% (Fig. 3A). Other known GH-regulated IGF ternary complex components, IGFBP3 and ALS, were also markedly reduced in GHRLD mice. GH was
increased ~4 fold, presumably the result of lack of feedback inhibition from IGF-1 (Fig. 3A). GHRLD mice had no difference in plasma glucose levels in a fed state (Fig. 3B),
but did display a modest increase in plasma insulin (Fig. 3C). Insulin tolerance tests (Fig. 3D) and glucose tolerance tests (Fig. 3E) revealed a mild diabetic insulin insensitivity.

<table>
<thead>
<tr>
<th>A.</th>
<th>GH (ng/mL)</th>
<th>IGF-1 (ng/mL)</th>
<th>IGFBP-3 (ng/mL)</th>
<th>ALS (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=15)</td>
<td>9.9 ± 0.7</td>
<td>395.9 ± 33.1</td>
<td>1083.2 ± 125.1</td>
<td>1992.6 ± 330.2</td>
</tr>
<tr>
<td>GHRLD (n=14)</td>
<td>33.0 ± 6.3</td>
<td>23.9 ± 4.2</td>
<td>220 ± 23.5</td>
<td>330.2 ± 41.7</td>
</tr>
<tr>
<td>p value</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Fig. 3. Liver-specific GHR deletion results in insulin resistance. (A) Serum measurements of GH, IGF-1, IGFBP3 and ALS in control and GHRLD mice. GHRLD mice had significantly higher plasma insulin levels in a fed state (C) with no change in plasma glucose (B). (D) Insulin resistance in response to 0.8U/kg IP insulin tolerance test. (E) Diabetic glucose tolerance after a 2gm/kgBW IP glucose challenge.

These mice also displayed a number of other metabolic derangements typically seen in the “metabolic syndrome” including dyslipidemia, elevated serum triglycerides, and hepatic steatosis (data not shown).
Gross examination of body weight, snout-to-tail body length, and tibia length showed no difference between controls and GHRLD mice (Fig. 4A). Micro-CT analysis of femurs from male 8 week old mice showed no significant differences between control and GHRLD animals in trabecular bone volume (Fig. 4B), trabecular number (Fig. 4C), mid-femoral cortical bone area (Fig. 4D), or mid-femoral cortical thickness (Fig. 4E).

RNA was harvested from distal femoral growth plates of male 8 week old mice and real-time PCR was performed examined IGF-1 mRNA levels. IGF-1 mRNA expression was increased in growth plates from GHRLD mice, although this increase did not reach statis-
tical significance (Fig. 5). Interpretation of this data is hampered by the heterogeneous nature of cell types contributing to RNA from the crudely dissected growth plate however. Further examination of the discrete changes in expression of IGF-1 and other growth factors in the growth plate of GHRLD mice is currently being performed using laser-capture microdissection.

In summary, the studies described in this dissertation project have provided valuable genetic models to study the GH/IGF-1 axis in bone. These model systems have allowed the first concise delineation of GH and IGF-1 effects, and will continue to prove useful in further studies of the mode of GH and IGF-1 anabolic actions in bone.

![Fig. 5. IGF-1 mRNA levels in the growth plate of GHRLD mice. Real-time PCR analysis of IGF-1 mRNA expression in femoral growth plates of 8 week old male control (n=7) and GHRLD (n=6) mice.](image-url)
GENERAL LIST OF REFERENCES


NOTICE OF APPROVAL

DATE: August 26, 2008

TO: Thomas L. Clemens, Ph.D.
VH-G001A 0019
FAX: 975-7410

FROM:

Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Defining Growth Hormone Action in Bone
Sponsor: NIH
Animal Project Number: 080807591

On August 26, 2008, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>A</td>
<td>582</td>
</tr>
</tbody>
</table>

Animal use is scheduled for review one year from August 2008. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 080807591 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: August 20, 2008
TO: Thomas L. Clemens, Ph.D.
   VH-9001A 6019
   FAX 975-7410

FROM: Judith A. Kapp, Ph.D., Chair
       Institutional Animal Care and Use Committee

SUBJECT: NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on August 28, 2008.

Title of Application: Defining Growth Hormone Action in Bone
Fund Source: NIH

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW) (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).