The Relationship Between Bone and Reproductive Hormones

Beyond Estrogens and Androgens

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Abstract

Reproductive hormones play a crucial role in the growth and maintenance of the mammalian skeleton. Indeed, the biological significance for this hormonal regulation of skeletal homeostasis is best illustrated by common clinical reproductive disorders, such as Primary Ovarian Insufficiency, Hypothalamic Amenorrhea, Congenital Hypogonadotropic Hypogonadism and Early Menopause, which contribute to the clinical burden of low bone mineral density and increased risk for fragility fracture. Emerging evidence relating to traditional reproductive hormones and the recent discovery of newer reproductive neuropeptides and hormones has deepened our understanding of the interaction between bone and the reproductive system. In this review, we provide a contemporary summary of the literature examining the relationship between bone biology and reproductive signals that extend beyond estrogens and androgens, and include kisspeptin, gonadotropin releasing hormone, follicle stimulating hormone, luteinizing hormone, prolactin, progesterone, inhibin, activin and relaxin. A comprehensive and up-to-date review of the recent basic and clinical research advances is essential given the prevalence of clinical reproductive disorders, the emerging roles of upstream reproductive hormones in bone physiology, as well as the urgent need to develop novel safe and effective therapies for bone fragility in a rapidly ageing population.

Keywords: Bone, Kisspeptin, GnRH, FSH, LH, Prolactin, Progesterone, Inhibin, Activin, Relaxin
Effects on skeletal homeostasis:
- Positive
- Negative
- No effect or uncertain
Introduction

Skeletal homeostasis in mammals is tightly regulated by the process of bone remodeling that preserves optimal bone mass and strength, thereby preventing fractures during normal physical activity. During bone remodeling, bone mass is maintained by a tight balance between osteoclastic bone resorption and osteoblastic bone formation. Furthermore, this bone remodelling is a considerably energy-demanding process as clearly demonstrated in rodents studies (1). Indeed, from an evolutionary perspective, during the physiological response to starvation, skeletal integrity as well as reproduction may be relinquished (2), whereas conversely food consumption is regarded as a positive stimulus for both bone (3) and reproduction (4). How bone remodelling is coupled specifically to energy metabolism has been the focus of several seminal studies revealing the importance of leptin in the interplay between bone and the central nervous system (5–7). Beyond leptin, other factors linked with nutrient intake and energy metabolism are suggested to regulate bone remodelling, including gastrointestinal hormones such as GLP-1, GLP-2 and GIP (8,9), as well as adipose-tissue factors such as adiponectin (10).

In skeletal diseases, bone remodelling is frequently disrupted. Osteoporosis, the most prevalent metabolic bone disease, is characterized by a deficit in osteoblastic bone formation relative to osteoclastic bone resorption, resulting in loss of bone mass and micro-architectural deterioration, increased bone fragility and resultant susceptibility to fractures (11,12). Post-menopausal bone loss is a central risk factor for developing osteoporosis (13,14). The higher risk and prevalence of fractures results in disability, poor quality of life and increased mortality (15). Notably, the rising incidence of osteoporosis (16) and health-care costs associated with an ageing society (13), accentuates the need to better understand bone physiology and the pathogenesis of bone loss.
It is well-recognized that skeletal homeostasis depends on the traditional hormonal mediators of calcium and phosphate homeostasis, such as parathyroid hormone (PTH), and vitamin D (17). Interestingly, co-expression of vitamin D receptor and vitamin D metabolizing enzymes in animal and human testis has been reported (18,19), suggesting that vitamin D could be considered a partly gonadal-derived factor, which influences bone. Moreover, while vitamin D may be formed and secreted from the testes, small animal and human studies reveal that active vitamin D promotes testosterone and sperm production, highlighting that vitamin D also acts indirectly on bone by promoting testosterone production in the testes (20). In addition, numerous other circulating factors with different primary roles are important in bone physiology, in particular reproductive hormones. Indeed, the importance of the crosstalk between reproductive hormones and bone is clearly illustrated by common reproductive disorders, which contribute to the clinical burden of low bone mineral density, such as Primary Ovarian Insufficiency (21–25), Hypothalamic Amenorrhea (26–28), Congenital Hypogonadotropic Hypogonadism (29–32), Pregnancy and Lactation-associated Osteoporosis (33–38) and Hyperprolactinemia (39–44) (Summarized in Table 1).

It was traditionally understood that the skeletal consequences of these disorders were primarily attributable to the effects of sex steroids (the gonadal reproductive hormones, oestrogen and testosterone). However, a decline in bone density during the perimenopausal transition frequently occurs despite unchanged circulating estrogen levels (45). Similarly, in rats, ovariectomy plus hypophysectomy results in less bone loss than ovariectomy alone underscoring a role for upstream reproductive hormones (46,47). Finally, whilst the prevalence of fractures in women with prolactin-secreting pituitary adenomas is high, this risk has been observed to be similar between amenorrhoeic and eugonadal pre-menopausal women, suggesting that hyperprolactinemia directly causes bone loss regardless of associated hypogonadism (44). Taken together, these data reveal that additional hormonal factors
contribute to the changes in bone mass in these reproductive disorders, rather than solely caused by changes in sex steroids. To this end, there is accumulating evidence identifying the importance of reproductive hormones of the hypothalamic-pituitary-gonadal (HPG) axis beyond estrogens and androgens in modulating key processes in skeletal homeostasis.

Notably, there are numerous existing reviews that comprehensively examine the effects of gonadal reproductive hormones (principally estrogen and androgens) on bone (48–52). Moreover, adrenal androgens (such as dehydroepiandrosterone, dehydroepiandrosterone sulfate and androstenedione), which act as precursors for peripheral conversion to more potent androgens and estrogens, also have established effects on bone homeostasis (53–58).

In this review, we provide a contemporary summary of the literature examining the relationship between bone and reproductive signals beyond these estrogens and androgens, including the recent basic (in vitro and in vivo) and clinical research advances, and the new players in the field. While several of the discussed hormones merit a review on their own, we have endeavored to combine them into a single atlas to provide an overall view of the relationship between reproductive hormones with bone beyond estrogens and androgens. A comprehensive and up-to-date review is essential given the prevalence of clinical reproductive disorders, the emerging roles of reproductive hormones beyond estrogens and androgens on bone physiology, as well as the urgent need to develop novel, safe and effective therapies for low bone mass to prevent bone fractures in an ageing population.
Methods

We performed a literature review and identified pertinent publications by a series of PubMed searches for English-language articles. The search terms were (“kisspeptin” OR “KISS1” OR “gonadotropin-releasing hormone” OR “GnRH” OR “luteinizing hormone” OR “LH” OR “follicle stimulating hormone” OR “FSH” OR “prolactin” OR “PRL” OR “progesterone” OR “inhibin” OR “activin” OR “relaxin”) AND (“osteocyte” OR “osteoblast” OR “osteoclast” OR “skeletal/skeleton” OR “bone” AND (“metabolism” OR “physiology” OR “structure” OR “remodelling” OR “modeling” OR “homeostasis” OR “tissue”) OR “bone mineral density” OR “osteoporosis” OR “fracture”). Relevant data were subsequently extracted from the identified publications, and secondary data sources identified therein. To ensure the inclusion of the most current data available, searches were performed up until the 21st of October 2020.

The Hypothalamic-Pituitary-Gonadal (HPG) Axis: A Historical Perspective and Overview

Reproduction is governed by the HPG axis, which acts as a classical negative feedback loop to regulate gonadal function. Indeed, it was Geoffrey Harris’ 1955 monograph 'Neural Control of the Pituitary Gland' which first posited that the secretion of pituitary gonadotropins was controlled by chemical substances of hypothalamic origin released into the hypophysial (pituitary) portal circulation (59). Consequently, this conceptual groundwork led to the isolation and sequencing of GnRH by the laboratories of Schally (60) and Guillemin (61) in 1971, and their award of the 1977 Nobel Prize in Physiology or Medicine. For decades GnRH would be considered the key determinant of reproductive function.

However, in 2003 two independent groups published reports in short succession revealing that humans with inactivating mutations of the kisspeptin receptor (GPR54/KISS1R) resulted in failed puberty and resultant infertility (62,63). These landmark findings in reproductive biology
were followed by a series of studies demonstrating that kisspeptin administration acted as a potent stimulator of gonadotropin release in animals (64–67) and humans (68–70), whereas pre-treatment with a GnRH antagonist abolished this stimulatory effect from kisspeptin (71).

In light of such fundamental discoveries, the historical understanding of the hormonal cascade which controls the HPG axis was revised (Figure 1): kisspeptin sits at the apex of the reproductive axis and is secreted by hypothalamic kisspeptin neurons. Kisspeptin activates kisspeptin-receptors expressed on GnRH neurons to secrete GnRH into the local hypophyseal-portal circulation in a pulsatile manner. Subsequently, GnRH is responsible for stimulating the biosynthesis and secretion of gonadotropins (LH and FSH) from the anterior pituitary gland, which circulate systemically to reach the gonads to promote gamete maturation and the release of sex steroids (estradiol, testosterone and progesterone). In addition to these established members, gonadal-derived activin and inhibin are closely related reproductive hormones with diametrically opposing biological effects: activin enhances FSH secretion, whereas conversely inhibin inhibits FSH secretion.

**Bone Modeling and Remodeling: A Brief Overview**

Bone modeling is a bone maintenance mechanism mediated by osteoclastic bone resorption followed by osteoblastic bone formation, which are coupled in time and space. Under steady state, there is a balance between bone resorption and bone formation and thus stable bone mass. Osteoclastic and osteoblastic functions are regulated by circulating extrinsic factors or locally by osteocytes. These are outlined below for context [and reviewed comprehensively in (72)].

**Osteocytes** comprise over 90% of bone cells and are the longest-lived bone cell, surviving for several decades, compared with days or weeks for osteoclasts and several months for
osteoblasts (73). They are derived from a subpopulation of mature osteoblasts that during the bone formation phase become embedded within mineralized bone matrix and function as the primary skeletal mechanosensors (74). RNA-sequencing analysis over the course of osteoblast to osteocyte transition using an osteoblast-like murine cell line has revealed significant changes in gene expression, with these changes associated with notable epigenic modifications to histones H3 and H4 (75). Importantly, these alterations are likely to be influential in determining the osteocyte phenotype (75). Functionally, osteocytes are predominantly responsible for modulating bone remodeling by regulating osteoclast and osteoblast differentiation through the release of several specific molecules, including sclerostin, receptor activator of nuclear factor-κB ligand (RANKL), osteoprotegerin (OPG) and dickkopf-1 (DKK1) (73,76).

Osteoclasts are multinucleated cells responsible for bone resorption and are derived from hematopoietic mononuclear cells of the monocyte/macrophage lineage (77) and have recently been demonstrated to recycle via daughter cells known as osteomorphs (78). Differentiation of the precursor cells into osteoclasts is primarily regulated by macrophage colony-stimulating factor (M-CSF) and RANKL. M-CSF acts on osteoclasts through its receptor c-FMS to induce the proliferation and survival of osteoclast precursor cells through the activation of ERK and Akt (79). By comparison, RANKL binds to its receptor, RANK, leading to the recruitment of adaptor molecules, such as tumour necrosis factor receptor-associated factor 6 (TRAF6), which subsequently leads to the activation of mitogen-activated protein kinases (MAPKs), and the transcription factors nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) (80). Activated NF-κB induces the nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), a major osteoclastogenesis regulator (80). Meanwhile, depending on their state of differentiation, osteoblasts (see below) transiently release RANKL and OPG. OPG functions as a decoy receptor for RANKL, to prevent the activation of RANK and thereby inhibit osteoclast
recruitment (81). In addition, osteocyte apoptosis with secondary necrosis results in release of damage-associated molecular patterns that induces osteoclastogenesis and bone loss (82,83).

**Osteoblasts** are mononucleated cells that are derived from bone marrow skeletal stem cells (also known as stromal or mesenchymal stem cells (MSC)). The commitment of MSC towards the osteoprogenitor lineage is regulated by a network of pro-osteogenic mediators, including bone morphogenetic proteins (*BMPs*) and members of the Wingless (*Wnt*) family (84). In addition, other important genes involved in this osteoblast differentiation include runt-related transcription factors 2 (Runx2), distal-less homeobox (Dlx5) and osterix (Osx) (84). During bone formation, mature osteoblasts synthesize and secrete bone matrix proteins, such type 1 collagen and several non-collagen proteins such as osteocalcin (OC), osteopontin and bone sialoprotein (84). Osteoblasts therefore mediate bone formation through the deposition of unmineralized osteoid matrix and its mineralization (85).

Each of these cell types are regulated by a variety of endocrine factors. In addition to established hormonal mediators, such as PTH, vitamin D, calcitonin, thyroid hormone, growth hormone, insulin-like growth factor 1 (IGF-1) and glucocorticoids (72,86,87), a wealth of emerging studies highlights the importance of reproductive hormones, as detailed in the current review. Therefore, we have considered the key components of the HPG axis (starting from the top) beyond estrogens and androgens, with regards to *in vitro* as well as non-human/human *in vivo* perspectives to ensure clarity and full appreciation of the important differences between these methodologies and the implications of their findings.
Kisspeptin

Kisspeptin refers to a family of structurally related endogenous peptides encoded by the human KISS1 gene (non-human Kiss1 gene). Diverging in amino acid length, they are the proteolytic products of a common 145-amino acid precursor protein (88,89). Four circulating fragments have been identified in the human circulation: kisspeptin-54, -14, -13 and -10 (with suffix denoting the number of amino acids) (89). All have a common RF-amide C terminus, which acts as the endogenous ligand for a G protein-coupled receptor, the kisspeptin receptor (KISS1R/Kiss1r) (90). Whilst the KISS1 gene was initially identified for its ability to reduce the metastatic potential of malignant melanoma cells (91), subsequent studies illustrated the indispensable influence of kisspeptin-signaling in pubertal progression and fertility (62,63,92). Interest has further accelerated in response to contemporary work highlighting a more expansive role for kisspeptin in the control of human reproductive behaviour, mood and emotions (93–96).

From a skeletal perspective, loss of function in the kisspeptin-signaling pathway in humans is associated with delayed skeletal maturation (62), whereas conversely activating mutations in KISS1R produces a phenotype of accelerated growth and skeletal maturation due to central precocious puberty (97). However, in both cases the direct contribution of kisspeptin on the skeleton cannot be adequately assessed, due to the respective large decreases and increases in gonadal sex steroids resulting from these mutations.

In humans, notable peripheral KISS1R expression has been detected in the heart, kidney, lung, pancreas, placenta, small intestine, spleen, stomach, testis and thymus (88,89,98). Within the hypothalamus, the distribution of kisspeptin-expressing neurons varies in a species-dependent
manner. In humans, two principle populations exists, (I) infundibular nucleus (II) rostral pre-optic area (99,100). In rodents, kisspeptin exists predominantly in two distinct populations: (I) arcuate nucleus (equivalent to the human infundibular nucleus) and (II) anteroventral periventricular nucleus (71,101,102). The detection of kisspeptin-signaling in bone cell lines (103) may imply its putative role in bone physiology as detailed below:

**In Vitro Studies**

Both KISS1 mRNA and protein are strongly expressed in immortalized human fetal osteoblastic cells transformed by expression of SV40 large T antigen (hFOB1.19) (103). By contrast, KISS1 mRNA and protein expression are moderate, weak and almost lost in the osteosarcoma cell lines U-2 OS, Saos-2 and MG-63, respectively (103). However, interestingly this did not match human osteosarcoma specimens where 20 of the 44 specimens exhibited strong KISS1 expression by immunohistochemistry, positively correlating with earlier distant metastasis compared with KISS1 negative patients (103). Consistent with this, the KISS1R protein product is highly expressed in MG-63 osteoblast-like osteosarcoma cells (104). Furthermore, Kiss1r expression has also been variably detected in normal canine osteoprogenitor cells (i.e. committed but not differentiated osteoblastic cells) (105) and human osteoprogenitor and skeletal stem cells (106). Further work is required to determine the precise expression pattern of KISS1 and its receptor in healthy mature primary bone cells.

Building on these observations, a recent study provided the first data for the direct role of kisspeptin in osteoblast differentiation. In C3H10T/2 mouse mesenchymal stem cells, kisspeptin-10 dose-dependently induced the expression of osteoblastic marker genes including Dlx5, Runx2 and alkaline phosphatase (ALP) (107). Given that BMP-2 stimulates bone formation by regulating the transcription of these osteogenic genes (108,109), the investigators subsequently demonstrated that kisspeptin-10 increased BMP-2 gene and protein expression,
via the transcriptional factor NFATc4 (107). Conversely, in kisspeptin-receptor null cells, osteoblast differentiation was suppressed (107). Hence, these data illustrate that in C3H10T/2 cells kisspeptin-10 (acting via Kiss1r) stimulates osteoblast differentiation through NFATc4-mediated BMP-2 expression and activation (107), which suggests a potential osteoanabolic role for kisspeptin in bone physiology.

In addition to BMP-2, kisspeptin signaling also regulates the expression of BMP-7 (another osteogenic gene) through the cooperative effect of the transcription factors NFATc2 and Sp1 in the embryonic kidney (110). Notably, Kiss1r deletion resulted in decreased BMP-7 expression and abnormal kidney branching morphogenesis and glomerular development in vivo and in explanted kidneys in vitro (110). Similarly, the mutual interaction of kisspeptin, estrogen and BMP-4 has also been identified to regulate GnRH production in mouse hypothalamic GT1-7 cells (111). Critically, whether the interaction between kisspeptin and BMP-4 or -7 affects skeletal morphogenesis remains to be elucidated.

KISS1R has also been detected in osteoclast cell lines differentiated in vitro from CD14-selected monocytes (112), suggesting that kisspeptin signaling may have direct roles not only in osteoblast but also osteoclast physiology, again highlighting the need for the assessment of KISS1/KISS1R expression in mature primary bone cells. Whether kisspeptin and its receptor are expressed in osteocytes and influence secretion of factors involved in remodelling (such as sclerostin) remains unknown and will no doubt be a focus of future investigation.

**In Vivo Non-Human Studies**

A contemporary pivotal murine study using a combination of different genetic models and stereotaxic surgery, demonstrated that deleting estrogen receptor alpha (ERα)-signaling in the hypothalamic arcuate nucleus resulted in a significant increase in bone mass without changes to food intake (113). In this study, the effect was sex-specific (occurring in female mice only).
with an impressive increase in trabecular bone mass of ~700% with an average 80% increase in bone volume over total volume (BV/TV) (113). Moreover, an increase in trabecular number and thickness, as well as increased overall mechanical strength of long bones was observed, in the absence of significant changes in several measured circulating hormones including leptin, thyroxine, LH, FSH, testosterone and estrogen (113). Mechanistically, these changes were accompanied by a significant increase in bone formation rate and mineralized surface, indicating enhanced osteoblastic functions (113). Transcriptional profiling demonstrated upregulation of BMP signaling and osteoblast differentiation (113). Remarkably, ablation of arcuate ERα after ovariectomy resulted in a 50% increase in bone density, indicating that even in the absence of gonadal hormones, the brain circuit remains partially intact (113) and suggest a possible therapeutic avenue for post-menopausal loss of bone mass. In addition, loss of ERα specifically in kisspeptin-expressing arcuate cells recapitulated this bone phenotype, defining central kisspeptin-signaling as a key mediator in ER-neuroskeletal circuit (113). Importantly, arcuate kisspeptin neurons are well-established as major neurons involved in coordinating energy states with reproduction (114). Therefore, it is interesting to speculate that these kisspeptin neurons are part of a wider system that controls energy-demanding bone remodeling in order to maintain reproduction.

Based on the above KISS1/KISS1R expression in vitro data (Figure 1), whether peripheral kisspeptin-signaling may also have direct beneficial roles in bone physiology in vivo remains to be seen. Along these lines, future investigation should seek to determine the skeletal consequences of conditional deletion of KISS1/KISS1R in bone cells as well as the effects of peripheral kisspeptin administration to examine this further and potentially open up new therapeutic avenues.
Gonadotropin-Releasing Hormone (GnRH)

The decapeptide GnRH is produced by neurosecretory GnRH neurons within the preoptic area and mediobasal hypothalamus and is released in synchronized pulses into the local hypophysial-portal circulation (115). Thereafter, it binds to its high affinity seven-transmembrane G-protein coupled receptor, GnRH receptor (GnRHR), expressed at the cell surface of gonadotropin cells of the anterior pituitary gland, and signals through a $G_{q/11}$-dependent intracellular pathway to control both the biosynthesis and secretion of the two gonadotropins (LH and FSH) (116).

In many vertebrates, three forms of GnRH (GnRH I, II, and III) have been identified, although only two exist in reptiles, birds and mammals (117). Correspondingly, three cognate receptor subtypes (types I, II and III) are present in amphibians, whereas in mammals only type I and type II are found (117). Indeed, GnRH II is widely distributed in the nervous system, and has been detected in normal and cancerous human tissues, including breast, endometrium, ovary and prostate (118), as well as bone marrow (119). Based on the latter, its potential involvement in bone physiology has been examined as reviewed below.

In Vitro Studies

Whilst data from primary cell culture is lacking, a recent study provided potential evidence for direct effects of GnRH on osteoblast-like cells. In both canine osteosarcoma cell lines (COS, POS, HMPOS, D17 and C4) and to a lesser extent in normal canine osteogenic progenitor cells, GnRH and GnRHR expression were observed (105). Furthermore, using the tumour cell line COS, detectable concentrations of GnRH were identified using radioimmunoassay (105), suggesting that these osteoblast-like cells can secrete GnRH in measurable amounts. Critically,
as these are osteosarcoma cells, this may reflect epigenetic changes. Remarkably, exogenous kisspeptin-10 applied to COS cells stimulated GnRH secretion by 4-5 fold (105), therefore recapitulating within these cells the normal functional relationship observed in the hypothalamic component of the HPG axis. In these studies, GnRH (and kisspeptin) treatment increased both COS proliferation and the expression of the bone remodeling ligand RANKL (but not OPG expression), effects which were blocked by treatment with a GnRHR inhibitor (105). GnRH and kisspeptin-10 treatment also increased the expression of the serotonin receptor htr2a by 2-8fold (105). Interestingly, the serotonergic system has been reported to regulate bone mass via osteoblast recruitment and proliferation (120) and suggests that GnRH and kisspeptin may exert osteoblastic pro-proliferative effects in these osteosarcoma cells. These results, although based on non-healthy (osteosarcoma) cell lines, provide an interesting insight into a possible role for GnRH on bone remodeling, potentially through interplay with the serotonergic system (Figure 1). Owing to the short half-life of GnRH of 2-6 minutes in vivo caused by high renal clearance and proteolytic degradation (121), there unfortunately remains a paucity of data examining the direct influence of GnRH on skeletal metabolism using in vivo models. However, cell-specific gene targeting may provide some answers, for instance by generating a GnRHR floxed mouse with osteoblast specific deletion of the GnRHR to examine the direct effect of GnRH on bone. Moreover, it is unknown whether GnRHR is expressed in osteocytes, which warrants further investigation.

*In Vivo Human Studies*

Given that GnRH is released into the local hypophysial-portal circulation and has a very short half-life as mentioned above, circulating levels cannot currently be analysed in peripheral blood, making clinical studies challenging. Conversely, a plethora of studies have examined the effect of GnRH agonists on bone mineral density (BMD) during therapeutic use as they suppress gonadal function and cause hypogonadotropic hypogonadism. For instance, androgen-
deprivation therapy remains the backbone of management for patients with prostate cancer, with GnRH agonists as the most widely used first-line treatment (122). In a study of 47 men with advanced or recurrent prostate cancer and no bone metastases, treatment with the GnRH agonist leuprolide was associated with 2-3% reduction in lumbar spine, trochanteric and total hip BMD, as well as a decrease in trabecular BMD by 8.5% after treatment for 48 weeks (123). In keeping with these data, most studies report a 2-3% decrease per year in BMD of spine and hip during initial GnRH agonist treatment (124). These significant changes in BMD result in a clinically relevant increased fracture risk. In men surviving at least five years after prostate cancer diagnosis, 19.4% of those who received androgen-deprivation therapy (orchidectomy or GnRH agonist) experienced a fracture (most commonly femoral neck, rib, spine and hand), as compared with 12.6% of those who did not (P<0.001) (125). Moreover, the risk of fracture increased with the number of doses administered during the first year after diagnosis (125). It is significant to note that novel GnRH antagonists (such as degarelix) are increasingly available for the treatment of advanced prostate cancer (126). Compared with GnRH agonists, they produce rapid and sustained suppression of testosterone without eliciting an initial testosterone surge (127). However, whereas the skeletal consequences associated with GnRH agonists are now well characterized, less is known regarding the bone effects of GnRH antagonists in patients with prostate cancer. In fact, a recent meta-analysis of randomized controlled trials in patients with metastatic disease reported that GnRH antagonists use was associated with fewer musculoskeletal events (relative risk 0.76, including fractures) compared with GnRH agonists (128). However as the authors rightly conclude, given the low number of musculoskeletal events and fractures observed, caution should be applied when interpreting these findings (128). Therefore, further clinical trials examining BMD and fracture risk are warranted before definitive conclusions regarding the effects of GnRH agonists versus antagonists on bone, can be drawn.
In addition to prostate cancer, GnRH agonists are also commonly employed as a treatment strategy to suppress ovarian function in the management of both endometriosis (129) and pre- and peri-menopausal women with breast cancer (130). In an analysis of 50 women with endometriosis, treatment with leuprolide administered for 24 weeks resulted in -4.9% and -3.4% reductions in BMD following 6 months of treatment and at 12 months of post-treatment, respectively (131). Similar reductions in BMD have also been observed in women with breast cancer. In a small multicenter study of premenopausal women with breast cancer, 2 years of goserelin alone caused a mean 5% loss of bone density, whereas combination with tamoxifen resulted in a lesser decline of -1.4% (132). It is notable that only partial recovery from bone loss was observed upon cessation of goserelin treatment alone at one year (132). Given these findings, the effects of concomitant treatment with bisphosphonates on preventing bone loss associated with GnRH agonists was examined. In the ABCSG-12 study, after 3 years of treatment in premenopausal women with endocrine responsive breast cancer, endocrine therapy alone (goserelin and anastrozole or goserelin and tamoxifen) resulted in a significant reduction of BMD of lumbar spine (-11.3%) and trochanter (-7.3%) (133). Again, only partial recovery was observed 2 years after completing treatment (133). By comparison, patients who received zoledronic acid had stable BMD at 3 years and increased BMD at 5 years (133), suggesting that concurrent bisphosphonate treatment has the potential to attenuate bone loss associated with GnRH agonists in this patient group.

Taken together, these studies indicate that bone remodeling is likely to be affected by GnRH agonist therapy (and probably GnRH antagonists), resulting in bone loss. In addition, from a mechanistic standpoint, GnRH agonist treatment of premenopausal women with endometriosis has been observed to result in osteocyte apoptosis in human bone, providing a further cellular mechanism for the increased bone fragility associated with these agents (134). However, although these results do not necessarily indicate an absence of the direct effect from GnRH on
bone physiology per se, the observed deleterious effects on the skeleton are most likely resulting predominantly from suppressing the release of subsequent downstream pituitary and gonadal hormones which have more established effects on bone.

**Follicle-Stimulating Hormone (FSH)**

FSH is synthesized by gonadotrope cells in the pituitary and plays a key role in mammalian reproduction during puberty and gamete production in adulthood. In women, FSH is responsible for follicular development and estrogen production (135), whereas in men FSH regulates testicular development and spermatogenesis (136).

FSH is a glycoprotein dimer consisting of an alpha (α) and beta (β) subunit. Whilst the α-subunit is common to thyroid stimulating hormone (TSH), human chorionic gonadotropin (hCG) and LH, the β-subunit is unique to FSH. This allows binding to its cognate receptor, the FSH receptor (FSHR), which belongs to the family of G protein-coupled receptors (137). In addition to the canonical Gsα/cAMP/PKA signaling pathway, it is now clear that FSHR activation triggers numerous other intracellular signaling pathways to elicit its biological actions (138).

Whereas FSHR was traditionally accepted to be exclusively localized in the gonads (137), recent studies have identified its expression in a variety of healthy extragonadal tissues, including placenta, umbilical cord vessels, uterus, liver and bone (139). Hence, interest into the putative direct actions of FSH on bone has flourished, particularly in view of early observations that a decline in bone density occurs during the perimenopausal transition when circulating FSH levels are markedly raised despite preserved estrogen levels (45).
In Vitro Studies

FSHR mRNA has been detected in murine and human osteoclasts and marrow skeletal stem cells, but not in mature osteoblasts or fibroblasts (140). Consistent with this, FSH (but not LH) stimulates osteoclastogenesis from human mononuclear cell precursors in a dose-dependent manner (140). Moreover, FSH stimulates the expression of the differentiation marker tartrate-resistant acid phosphatase (TRAP), but does not affect precursor proliferation, indicating that FSH may preferentially influence the differentiation rather than the proliferation of osteoclast precursors (140). Indeed, FSH upregulates three established osteoclastogenic pathways by enhancing the phosphorylation of Erk1/2, IκBα, and Akt to simulate osteoclast formation (140).

Furthermore, FSH induces the expression of RANK on CD14+ human peripheral blood mononuclear cells (141). It is interesting to note that this occurs in a biphasic manner, such that FSH at a concentration of 10 mIU/ml (i.e. similar to circulating follicular-phase FSH levels of women during reproductive years) or 100 mIU/ml (i.e. FSH levels after menopause) had no significant influence on RANK expression (141). By comparison, at 50 IU/ml (i.e. typical during perimenopause), FSH significantly increased RANK expression (141), tentatively providing further evidence for the role of FSH as a stimulus for osteoclast differentiation, particularly during the perimenopausal transition.

In addition to directly enhancing osteoclastogenesis as above, FSH has also been implicated in modulating the activity of several pro-inflammatory cytokines involved in regulating osteoclastic bone resorption. Murine bone marrow cultures exposed to recombinant FSH stimulated tissue necrosis factor alpha (TNFα) production, resulting in an increase in the osteoclast precursor pool (142). In addition, isolated mononuclear cells from 36 premenopausal women incubated with exogenous FSH induced the mononuclear cells to secrete IL-1β (143).
In murine calvarial organ cultures using ex vivo calcein labeling, FSH did not increase calcein-labeled surface area, whereas BMP-2 as a positive control increased it by 6-fold (140). Mechanistically, this is consistent with absent FSHR on mature osteoblasts, suggesting that FSH modulates predominantly osteoclastic rather than osteoblastic activity. Moreover, FSHR in osteocytes remains to be examined.

**In Vivo Non-Human Studies**

To examine the influence of FSH on bone, the in vivo effects of deleting FSH or its receptor have been examined in mice. In FSHR null females, whereas areal and volumetric BMD at both trabecular and cortical sites were indistinguishable between the mutant and ovariectomised controls, the latter group demonstrated a 15% reduction in lumbar spine areal BMD by 8-weeks compared to the mutant mice (140). Furthermore, despite severe hypogonadism (as evidenced by atrophic ovaries and thread-like uteri), FSHβ deficient homozygous female mice did not lose bone, with both areal and volumetric BMD increased (140). Comparatively, FSHβ deficient-heterozygous females were eugonadal (as evidenced by normal ovaries and uteri) and fertile (with a 50% reduction in FSH levels), which was associated with an increase in spinal and femoral areal BMD (140). Although it may be tempting to speculate that FSH action is required for hypogonadal bone loss, it is worth bearing in mind that in this study, circulating levels of LH, estrogen, and testosterone were not reported. This is particularly relevant given that it is well-established that both LH and testosterone become elevated in FSHβ and FSHR null mice (144–146), which makes a definite conclusion about the direct role of FSH from these experiments somewhat uncertain. Therefore, to establish that complete loss of FSH-signaling in FSHβ and FSHR null mice protects from bone loss (despite severe hypogonadism), ovariectomy in the mutant mice (thus eliminating androgens) could have been useful in this regard (147), an experiment that was performed solely in the control mice in this study (140). Possible
experimental alternatives would include a genetic or chemical approach to inhibit androgen secretion or gene expression in the ovaries.

To examine the effect of blocking FSH on ovariectomy-induced bone loss in mice, a 13-amino-acid-long peptide polyclonal antibody that is directed to the receptor-binding domain of the β-subunit of FSH has been generated (148). The FSH antibody abolished FSH-induced osteoclast formation in vitro (148). Moreover, when administered to ovariectomized mice, it was effective in attenuating bone loss by stimulating bone formation and inhibiting bone resorption suggesting a possible therapeutic avenue (148).

By contrast to the above studies, further experimental data reveals that a direct role for FSH in bone remains unclear. By 3 months of age, a 4.9% and 5.6% reduction in femoral and lumbar spine BMD, respectively was observed in FSHR null mice (149). It is striking that these deleterious effects were rescued by allogenic ovarian transplantation, which increased circulating estradiol levels, and reduced LH and testosterone (149), suggesting that downstream ovarian function is more important for age-dependent bone loss in this model. Moreover, bilateral ovariectomy decreased elevated testosterone levels in FSHR null mice and reduced BMD to levels comparable with ovariectomized wild-type controls (149). Hence, to investigate whether elevated ovarian androgens contribute to the skeletal responses to ovariectomy, FSHR null mice were treated with the androgen receptor antagonist flutamide and the aromatase inhibitor letrozole (149). Notably, both resulted in bone volume reductions in these mice, suggesting that ovarian androgens as well as estrogens affect skeletal homeostasis independent of the action of FSH (149).

In keeping with these data, the effects of elevated FSH on bone mass and structure have been studied using transgenic female mice expressing human FSH (150), an experimental paradigm which results in rising circulating FSH levels with age (151). In this study, raised FSH induced
bone formation and increased bone mass, an effect which was observed to be dependent on ovarian function, but independent of GnRH or LH activity (150). Moreover, further experiments reveal that estrogen deficiency is the dominant factor impairing bone loss in ovariectomized wistar rats (152). Here, whilst FSH and LH were observed to modulate bone loss, changes in estrogen had a more powerful influence (152). Taken together, whilst a range of studies suggest a role for FSH in bone physiology, the reported effects may be (in part) mediated via gonadal-pathways. An additional limitation of the reported experiments is that they were limited to female (not male) mice models and studies in male mice are needed to examine for possible sexual dimorphisms. Approaches including FSHR floxed mice with osteoclast specific deletion of the FSHR may be helpful to definitively determine the physiological role of FSH in bone and reconcile the above findings.

**In Vivo Human Studies**

Numerous observational studies reported an association between rising serum FSH levels and bone loss. The multi-site, longitudinal Study of Women’s Health Across the Nation (SWAN) examined 2,375 pre- and early perimenopausal women of African-American, Caucasian, Japanese and Chinese background (153). In these pre- and early menopausal women, higher FSH concentrations (but not other serum reproductive hormone levels) were associated with higher concentrations of the bone turnover markers urinary N-terminal telopeptide of type I collagen (NTX) and serum OC before as well as after adjusting for covariates (including BMI, smoking status, physical activity and dietary intake variables, such as alcohol and calcium) (153). These observational findings suggest a possible relationship between increased levels of FSH and premenopausal increased in bone turnover. In a further observational study of 2311 pre- and early perimenopausal women, statistical modeling using the baseline FSH values and subsequent follow-up FSH levels, predicted the 4-year BMD reduction after adjusting for various factors (such as ethnicity and baseline age) (154). In this cohort, spinal and hip BMD reduction
during the menopausal transition was strongly associated with the initial FSH and follow-up FSH levels, but not with estradiol levels (154).

Given the putative relationship between both BMD and bone turnover with serum FSH in postmenopausal women, the influence of harboring certain polymorphisms in the FSH/FSHR system has been evaluated. Two hundred and eight-nine postmenopausal women were genotyped for the single nucleotide polymorphism (SNP) rs6166 in exon 10 of the FSHR gene (155). In this observational study, AA rs6166 women demonstrated lower BMD at the femoral neck and total body, along with higher serum levels of ALP and C-terminal telopeptide of type 1 collagen (CTx), compared with GG rs6166 women (155). Moreover, the prevalence of osteoporosis was significantly higher in AA rs6166, an effect which was shown however to be independent of circulating levels of FSH or estrogen (155). By comparison, in the largest meta-analysis of genome-wide association studies for BMD involving 32,961 individuals of European and East Asian ancestry, 56 genome-wide loci were associated with BMD of the lumbar spine and/or femoral neck, and 14 of those also associated with fracture risk in a case-control meta-analysis involving 31,016 fracture cases and 102,444 controls without fractures (156). This large genomics study did not identify any FSH-related signal, including in the FSHR gene. Importantly, future studies employing Mendelian randomization would be informative to further interrogate a possible causal relationship between FSH and bone.

In addition to bone loss associated with the menopausal transition, the bone effects of elevated levels of FSH observed in secondary amenorrhea in women of reproductive age has been investigated. In a small observational study of 22 amenorrhoeic and 12 eumenorrheic women under the age of 40, amenorrhoeic women had lower lumbar BMD (although no difference in femoral neck BMD) than eumenorrheic women (157). The amenorrhoeic women were then separated into two groups according to their FSH levels: hypergonadotrophic amenorrhea (i.e. FSH >40 IU/L) and hypogonadotrophic amenorrhea (i.e. FSH ≤40 IU/L) (157). The
hypergonadotropic women displayed a greater reduction in BMD than the hypogonadotropic women (157). Consistent with this, only FSH had a negative correlation with lumbar spine BMD in the hypergonadotropic group, whereas there was no correlation between FSH levels, BMI, age or duration of amenorrhea in the hypogonadotropic group (157). Importantly, the analysis was not adjusted for age, despite the hypergonadotropic women being 7.6 years older than the hypogonadotropic women (37.43 versus 29.8 years) (157), which may in part explain some of the differences in observed BMD between the groups.

By comparison, other observational studies do not reveal an association between FSH and bone. In a study involving 137 middle-aged infertile men (due to spermatogenic failure) and 70 aged-matched healthy men with normal fertility, 15 years after infertility work-up there was no difference in BMD between the two groups, despite a significantly higher median FSH value (9.8 versus 3.7 IU/L) (158). Importantly, total testosterone and estradiol were similar between the two groups at follow-up (158). Indeed, neither the baseline or follow-up FSH levels exhibited significant correlation with axial, femoral or total body BMD, indicating that infertile (but eugonadal) men with high FSH levels do not have lower BMD (158). In a similar cohort involving 307 men with idiopathic infertility and 28 men with Klinefelter Syndrome, serum FSH levels did not exhibit significant correlation with BMD, nor with the RANKL/OPG ratio, OPG, PTH or osteocalcin (159). Interestingly, FSH was inversely correlated with serum levels of soluble RANKL in both cohorts of men, an effect which remained significant after adjustment for relevant nonhormonal confounders (age, body fat percentage and smoking in the idiopathic infertility cohort, whereas age-adjustment only in the Klinefelter Syndrome cohort) and serum estradiol (159).

Critically, whilst numerous observational studies suggest an association between FSH and bone, only interventional studies are able to detect a reliable direct cause-and-effect relationship between FSH and bone turnover. Indeed, in a seminal prospective study involving 21 post-
menopausal women treated with the GnRH agonist leuprolide and 20 control women receiving placebo injections, both groups concurrently received the aromatase inhibitor letrozole to eliminate variations in endogenous estrogen levels (160). At 3.5 months, in response to GnRH agonist-induced suppression, serum FSH fell by 86% (into the premenopausal range), but did not change significantly in the control women (160). Notably, in the women receiving the GnRH agonist, suppression of FSH release resulted in larger increases in bone resorption markers than in controls (160). Furthermore, although there was also a small decrease in testosterone in the women administered GnRH agonist (21% reduction in an already low postmenopausal testosterone), it is unlikely to have masked any significant positive effects of FSH reduction on bone resorption. Taken together, this experimental model provides direct evidence that FSH does not modulate bone resorption markers in a postmenopausal woman.

In keeping with this interventional study, further experimental evidence in humans also suggests that FSH does not exert independent effects on bone physiology. In a study involving 29 infertile women undergoing in vitro fertilization, administration of the GnRH analogue leuprolide was followed by stimulation with recombinant FSH (rFSH) (161). In response to leuprolide-induced suppression of serum FSH and estrogen levels, bone turnover markers increased as indicated by a significant rise in serum β-CTX (161). Moreover, 3 days after the first dose of rFSH, despite serum FSH values above the reference range for the early follicular phase (with estradiol maintained in the reference range), no significant change in serum β-CTX was observed (161). By comparison, serum β-CTX was lower and FSH and estradiol levels higher 10 days after the first administration of rFSH (161). Therefore, in this experimental model, short term administration of rFSH did not exert any significant change in serum levels of bone turnover markers, which instead exhibited significant correlation with serum estradiol levels.

Given the experimental data examining the influence of FSH on bone turnover in both pre- and post-menopausal women, it is interesting to consider its effects in men. In a randomized
controlled trial involving eugonadal men, participants were treated with a monthly GnRH agonist (goserelin) and topical testosterone and compared with a control group receiving placebo (162). Importantly, participants in the intervention group were individually matched with participants in the control group to ensure the mean serum testosterone and estradiol levels achieved during the treatment period did not differ between groups, therefore eliminating the confounding influence of gonadal sex steroids (162). Following 16 weeks of treatment, serum FSH fell by 60% in the intervention group and 2% in the control group (162). Despite the substantial suppression of FSH levels in the intervention group, serum levels of biochemical markers of bone resorption (serum NTX and CTX) and bone formation (serum osteocalcin) did not change (162), suggesting that in the eugonadal range, FSH does not affect bone turnover in men. To this end, the putative role of FSH as a direct modulator of bone physiology (Figure 1) remains a controversial area (147). In vitro and in vivo animal studies have reported conflicting results regarding a direct role for FSH in the modulation of bone turnover. Given some of the inconsistencies in the literature, in vivo conditional deletion studies would be important to reconcile some of these discrepancies. In addition, while these assessments of bone turnover markers in shorter term interventional studies are highly informative, longer term (>6 months) in women and men with comprehensive additional bone assessments could be illuminating. Ultimately, whilst several (but not all) observational studies in humans have produced results suggestive of FSH effects on bone turnover, the nature of these studies does not prove causality. Indeed, to date, no interventional study in humans has detected a direct cause-and-effect relationship between FSH and bone turnover.
Luteinizing Hormone (LH)

The glycoprotein hormone LH is a heterodimer consisting of a non-covalently associated α-subunit common to several peptide hormones (including FSH and TSH) and a specific β-subunit conferring biological specificity (163). Belonging to the cystine knot superfamily (164), LH is secreted by the anterior pituitary gland at the time of pubertal onset to promote the maturation of the reproductive system in males and females and thereafter the secretion of the gonadal reproductive hormones. LH signals through a seven transmembrane domain G-protein coupled receptor, the LH receptor (LHR) (165), which is expressed in skin, mammary gland, placenta, uterus, urinary bladder, prostate, adrenals as well as osteoblast cell lines (166,167).

Furthermore, hCG is the placental homologue of LH and an additional ligand of the LHR (168), which represents the principal circulating gonadotropin during pregnancy. Importantly, given the established skeletal changes seen in puberty, pregnancy and menopause (169) (i.e. during periods of amplified levels of LH/hCG), may functionally imply that LH and hCG may participate directly in bone physiology as discussed below.

In Vitro Studies

Unlike FSH, there are fewer data about the effects of LH on bone. The presence of the LHR in extracts of primary human osteoblasts and osteoblast-like cell lines (mC3Ts-E1, MG63 and SAOS2) has been identified by Western blotting, immunolocalization and RT-PCR (167). However, stimulation of osteoblastic LHR with hCG did not increase downstream cAMP or ERK phosphorylation, raising the possibility that osteoblasts may actually express either low receptor numbers or nonfunctional receptors (167). In this study, the presence of LHR in osteoclasts and osteocytes were not reported.
By contrast, other investigators have observed that human osteoblasts treated with a urinary derived formulation of hCG, resulted in osteoblast proliferation as indicated by elevated ALP activity and increased MMP-2 expression (170). In fact, although hCG alone was capable of stimulating an increase in ALP activity, co-treatment with hCG and calcitriol resulted in a five-fold increase in ALP (170). In addition, in organ cultures of Ca\(^{45}\) labeled murine calvaria treatment with urinary hCG resulted in a dose-dependent release of Ca\(^{45}\) into the medium, suggesting a modest stimulation of osteoclastic bone resorption (170). Curiously, repeating these experiments with recombinant hCG (rather than urinary derived hCG), resulted in no change in osteoblast activity, which might indicate the presence of contaminating agents in urine derived hCG, an effect shown to be accounted for by the presence of epidermal growth factor (EGF) (170). Taken together, these data do not robustly support that hCG influences human osteoblasts, given there was no response when non-urine derived hCG was used. Moreover, whether LH/hCG directly influences osteoclast or osteocyte biology remains to be answered.

**In Vivo Non-Human Studies**

To explore the putative role of LH/hCG on the skeleton *in vivo*, an LH receptor null mutant (LuRKO) mouse model and a murine transgenic model overexpressing both hCG subunits (hCG αβ+) have been generated (167). Ablation of LHR resulted in 43% reduction in femoral BMD by 5 months of age and histomorphometric analysis revealed reduction in cancellous bone volume, trabecular width and number (167). Interestingly, 6-month male hCG αβ+ mice had comparable BMD to wildtypes, female hCG αβ+ mice exhibited ~30% increases in both tibial and femoral BMD suggesting the presence of sexual dimorphism (167). It is interesting to speculate as to whether the increase in BMD represented a consequence of reduced bone resorption and/or heightened bone formation, as well as a possible synergistic effect between additional ovarian
factors and increased levels of hCG. Hence, to determine if the observed increases in BMD was a direct effect of raised serum hCG, or an indirect ovarian effect, female hCG αβ+ and wildtype mice were bilaterally ovariectomized at 3-weeks of age, which resulted in 36% and 33% reductions in femoral and tibial BMD, respectively (167). Collectively, these data supports the role of the ovary in precipitating the increases in bone volume observed in hCG-overexpressing mice, thus confirming an indirect effect of LH on the skeleton (167). It is important to note that to date the in vitro and in vivo studies have involved the application of hCG (rather than LH itself due to LH’s much shorter half-life), since both hormones signal through the same receptor. An interesting future area of study would be to investigate whether hCG and recombinant LH have differential effects on bone physiology.

In Vivo Human Studies

The relationship between the level of serum LH and cytokines associated with skeletal homeostasis has been investigated in 694 healthy Chinese women (171). After adjusting for age, BMI and estradiol levels, serum LH showed no significant correlation with serum cytokine levels in premenopausal women, but exhibited a significant positive correlation with OPG and TGF-β2 in perimenopausal women (171). Additionally, in postmenopausal women, LH levels also showed a positive correlation with OPG levels, but not with TGF-β2 (171). These observations provide potential mechanistic insight into bone physiology during perimenopausal and postmenopausal periods (i.e. at times of amplified LH levels with concentrations up to 10 times of those found pre-menopause) although association does not indicate causality.

Nonetheless consistent with these findings, LH has also been shown to be positively correlated with levels of bone turnover indicators in the same cohort of Chinese women (172). Notably, whilst the influence of FSH was approximately seven to 20 times greater, LH was observed to
explain 2.1% and 1.1% of the changes in bone formation markers bone-specific ALP and OC, respectively, but had no apparent effect on bone resorption markers (172).

Whether these changes in cytokines and bone turnover markers exert or represent a deleterious influence on BMD, has been the focus of a plethora of studies. In a cross-sectional study by the same group, in healthy Chinese women (aged 20-82 years), serum LH negatively correlated with BMD at all skeletal sites and for each 10 IU/L increase in LH levels, BMD decreased by 4.4%, 2.8%, 3.6% and 2.4% at the posterior-anterior spine, lateral spine, total hip and radius/ultradistal, respectively (173). This finding was also observed in a later study in Mongolian women. In 260 women (aged 50.1 ± 4.4 years), serum LH was found to be higher in women with low BMD compared to women with normal BMD (174). It is notable that BMD was measured in the forearm and tibia, rather than more commonly assessed in the hip and spine (174). By contrast, additional studies have found no association between serum LH levels and BMD. In an analysis of 36 ovulatory women (aged 20-50) from the US, whereas serum FSH concentration was inversely related to BMD measures, LH was not (143). Similarly, no difference in serum LH levels was detected in 73 post-menopausal Turkish women with low versus normal femoral and lumbar BMD (175). Given the previous studies, it is possible that the latter two studies failed to detect an association due to their small sample sizes.

Studies have also examined the relationship between LH and BMD in men. In community-dwelling older men, a significant inverse association between longitudinal change in hip BMD and serum LH (unlike testosterone and estrogen) has been observed in both univariate and multivariate analyses, suggesting that higher LH levels at baseline was associated with greater bone loss at the hip over the subsequent 5 years (176). Correspondingly, higher LH levels correlated with increased hip and nonvertebral fractures in univariate models, an effect which did not remain significant after adjustment for age, BMI, smoking status, physical activity and comorbidity (176).
A recent study assessed the association between reproductive hormones and the incidence of fractures in 3307 community-dwelling Australian older men (aged 76.8 ± 3.5 years) over a median follow-up period of 10.6 years (177). Men who experienced any incident fracture had a higher LH and lower baseline testosterone than men who did not experience a fracture (177). However, once adjusted for age, medical comorbidities and frailty, a U-shaped association between plasma testosterone and fracture was apparent, whereas there was no association with LH (177). This suggests that whereas LH stimulates testicular testosterone production, that circulating testosterone (but not LH) appeared to determine fracture risk. However, the analysis was based on a single baseline blood sample, rather than serial hormones measurements over time.

Taken together, the relationship between serum levels LH and BMD has been inconsistent across different populations. It is important to recognize that as these are observational studies, causality cannot be established especially in the absence of conclusive mechanistic in vitro data. In addition, larger prospective studies are necessary to elucidate the relationship between LH and BMD in greater detail and to isolate the effects mediated directly by LH. Considering the data from in vitro and in vivo experiments collectively, it is likely that LH exerts an indirect effect on bone and its effects are mediated predominantly via the downstream gonadal sex steroids (Figure 1).

**Prolactin (PRL)**

PRL is a peptide hormone present in all vertebrates, with the mature peptide composed of 199 amino acids (178). It is synthesized and secreted by lactotroph cells of the anterior pituitary gland, and exerts its biological actions by binding to its receptor, the PRL receptor (PRLR), a type 1 cytokine receptor (179).
Beyond the pituitary, PRL is produced by many other cells and tissues, including several brain regions, lacrimal and sweat glands, thymus, lymph nodes, breast, spleen, skin, myometrium, decidual cells of the placenta and bone marrow (180). In the majority of these regions, the physiological role of prolactin remains to be determined. Evidence suggests that PRL participates in excess of 300 identified biological processes in various vertebrates, including lactation, reproduction, metabolism, osmoregulation, behavior, growth and development, and immune regulation and protection (181). However, patients with PRL-secreting pituitary adenomas typically present with symptoms attributable to a space occupying lesion, HPG axis dysfunction and/or galactorrhea (182), rather than impairments of the aforementioned functions suggesting a lesser relevant role.

In animals and humans, circulating PRL is significantly elevated during pregnancy and lactation, along with numerous other corresponding changes in circulating estrogens and progesterone. Notably, these physiological states are recognized to induce a maternal bone-resorptive state in order to provide the necessary calcium for fetal and neonatal skeletal growth and development (183). As such, the potential role of PRL in bone physiology has been the focus of several studies.

**In Vitro Studies**

Osteoblasts express PRLR (184), providing early evidence that prolactin may play a physiological role in bone physiology. In certain osteoblastic cell lines, such as human osteosarcoma cells, the expression of PRLR mRNA is strongly influenced by the presence of osteotropic factors, such as the physiological concentration of 1,25-(OH)\(_2\) vitamin D\(_3\) (185). In contrast to osteoblastic cells, evidence from rats reveals that osteoclasts and osteocytes do not express PRLR (186).
PRL is able to indirectly regulate osteoclastic activity through osteoblastic cells. Indeed, the mRNA expression of osteoblast-derived osteoclast-regulating factors has been studied using rat osteoblast-like UMR106 cells treated with PRL in various concentrations (187). mRNA expression of MCP-1 and Cox-2 were upregulated by ~2-3-fold in the presence of 200-500 ng/ml PRL (187). Interestingly, only higher PRL concentrations of 500 ng/ml (i.e. comparable to the average suckling-induced PRL surge) upregulated TNF-α and IL-1 by ~3-fold and ~2-fold, respectively, whereas MCSF and IL-6 mRNA expressions were unaffected by 100-500 ng/ml PRL (187). In addition to activating osteoclastic cells by osteoblast-secreted cytokines, PRL has also been observed to upregulate the expression of ephrin-B1 by ~2-fold after exposure to 300 ng/ml PRL (187). This latter finding suggests that PRL can also facilitate osteoblast-osteoclast communication through a direct cell-cell contact using the ephrin system in vitro (187). Moreover, MG-63 cell line, exposed to sustained pathological concentrations of PRL (up to ~1000 ng/mL), increased the RANKL/OPG ratio, triggering an increase in osteoclastic bone resorption (39). In addition to the effects of PRL-induced activation of osteoclasts through osteoblast-secreted factors, PRL has also been observed to suppress osteoblast formation itself. In MG-63 cell line treatment with PRL led to lower expression of ALP and OC mRNA, and a decrease in ALP activity (39). Collectively, these data reveal that hyperprolactinemia may act directly on bone to stimulate bone turnover, with increased bone resorption than formation through osteoblast-related mechanisms.

In Vivo Non-Human Studies

In keeping with in vitro models, in vivo animal experiments reveal a prominent role for PRL in calcium and bone homeostasis. During pregnancy and lactation, studies in rats revealed that PRL stimulates intestinal calcium absorption (188). In fact, long-term exposure to PRL (over a period of several days) in pregnancy and lactation induced specific changes in duodenal cells by increasing the expression of genes related to transcellular transport (such as TRPV5/6 and
calbindin-D_9k) and paracellular transport (such as claudin-3), thereby increasing calcium absorption (188). Remarkably, during suckling the transient PRL surge increased calcium absorption within 30 minutes to match calcium loss in milk. This effect of enhanced transcellular and paracellular calcium transport is mediated by phosphoinositide 3-kinase, protein kinase C, and RhoA-associated coiled-coil-forming kinase pathways (188). Taken together, these data reveal that PRL acts in part as a calcium-regulating hormone by stimulating calcium absorption during pregnancy, lactation and related suckling.

Congruous to its influence on intestinal calcium absorption, PRL also has demonstrable direct effects on bone. PRLR gene deficient mice exhibited delayed calvarial ossification (in 18.5-day old embryos) (184). In addition, adult PRLR gene deficient mice demonstrated a significant decrease in trabecular and cortical mineral apposition rates in long bones (tibia and femora), along with a 60% decrease in bone formation rate, but no significant changes in the number of osteoclasts (184). Collectively, this suggests a role in bone formation with limited effects on osteoclastic bone resorption. Notably, this model also resulted in major changes in the levels of calciotropic and reproductive hormones including elevated serum PTH levels, and reduced serum estradiol and progesterone (184). Hence, in addition to direct effects from PRL on bone, some of these observed effects may be attributable to secondary hormonal changes.

Indeed, the direct effects of prolactin on bone physiology are frequently difficult to dissect due to complex hormonal changes from hyperprolactinemia-induced hypogonadism in vivo (189,190). To overcome this, anterior pituitary allografts have been transplanted under the renal capsule of recipient female rats with or without ovariectomy (39). Within 15 days of transplantation, continuous PRL secretion from the ectopic pituitary glands resulted in PRL levels of 91 ng/mL (equivalent to pregnancy levels in rats), but without increasing other pituitary hormones due to the absence of upstream stimulatory hypothalamic signals (39). Whereas femoral BMD and bone mineral content were unaffected, histomorphometric studies indicated enhanced bone
resorption with decreases in bone volume and trabecular number, whereas trabecular separation, and the osteoblast and osteoclast surfaces were increased (39). Interestingly, the presence of high physiological PRL levels (i.e. 90-100 ng/mL) may have resulted in extra calcium from enhanced intestinal calcium absorption, therefore contributing to the observed increase in the mineralization process, which in turn preserved the BMD. Crucially estrogen supplementation did not restore the effect of estrogen deficiency in the pituitary allograft plus ovariectomy rats suggesting estrogen-independent effects of PRL (39).

When taking into consideration the data from both in vitro and in vivo experiments, it appears that PRL exerts direct effects on bone remodeling, such that during periods of hyperprolactinemia, bone remodeling is stimulated with increased bone resorption and possibly decreased bone formation. Although the above data suggests direct effects, the presence of other reproductive hormone-dependent effects is plausible. Future studies employing techniques such as cell-specific gene targeting (for example for the osteoblastic prolactin receptor) may help delineate the precise direct effects of PRL further.

In Vivo Human Studies

The relationship between hyperprolactinemia and bone loss secondary to increased bone resorption has been the focus of a plethora of human studies. In a small study of 20 hyperprolactinemic men, although the majority had low BMD, four patients were found to have normal BMD at both the lumbar spine and femoral neck (40). Serum OC levels were lower, whereas urinary NTX levels were higher than the reference range in all the hyperprolactinemic men (40), suggesting that alterations in bone turnover may occur even before changes in BMD becomes apparent in men with hyperprolactinemia. In a subsequent analysis, a significant negative correlation was found between serum OC and PRL levels and disease duration, and a significant positive correlation between the urinary NTX and PRL levels and disease duration.
Patients with hyperprolactinemia exhibit decreased bone mass at skeletal sites enriched with trabecular (such as the spine and hip) rather than cortical bone (such as the distal radius) (22,41,191). Consistent with this, additional studies of patients with hyperprolactinemia demonstrated that vertebral BMD decreases by 20-30%, while forearm BMD decreases by 2.5-10% (192). In addition, the deleterious effects on skeletal health are more marked when hyperprolactinemia develops at a younger age, owing to the decreased peak bone mass. In keeping with this, in a study comparing 20 patients with hyperprolactinemia with disease onset during adolescence and 20 patients with disease onset during adulthood, BMD was significantly lower in younger compared to older adult patients, both at the lumbar spine and femoral neck highlighting the important clinical need to address hyperprolactinemia in adolescence to ensure optimal peak bone mass acquisition (42).

The prevalence of skeletal fractures in patients with hyperprolactinemia has been investigated in a number of studies. In a series of 86 patients with prolactinomas, the excess fracture risk before diagnosis was observed to be 60% higher compared with healthy controls (193). Furthermore, in a study of 32 men with prolactinomas (10 with microadenomas and 22 with macroadenomas), vertebral fractures were identified in 37% of men, compared with 8% of age-matched healthy controls (43). In fact, bone fractures occurred more frequently in those patients with a longer duration of disease and in patients with untreated hyperprolactinemia (43). Interestingly, the prevalence of vertebral fractures was not different between eugonadal and hypogonadal patients, nor was there a difference in serum testosterone between fracture and non-fracture groups (43), suggesting a gonadal-independent effect on fracture risk.

Consistent with the high prevalence of radiological vertebral fractures in men with prolactinomas, parallel studies in women reveal a similar susceptibility to skeletal fracture. In a cross-sectional study of 78 women with prolactinomas, vertebral fractures occurred in 32% of patients, compared with 13% of age-matched healthy controls (44). Patients with fracture were
older, had lower BMD, longer duration of disease, higher serum PRL and lower IGF-I levels as compared to women without bone fracture (44). Similar to men, bone fractures occurred more frequently in women with untreated hyperprolactinemia compared with patients treated with dopamine agonists (44).

Adequate treatment of hyperprolactinemia and resultant hypogonadism rescues bone loss, however recovery of BMD is only partial. In a study of 20 hyperprolactinemic men, despite restoration of testosterone and suppression of PRL, serum OC levels were normalized, whereas neither urinary NTX levels nor BMD values were normalized after 18 months of treatment with dopaminergic agents (40). Importantly, despite correction of serum PRL levels after 6-12 months of medical treatment, the improvement in BMD at both 12- and 24-months of treatment remained reduced in patients with disease onset during adolescence than onset during adulthood (42).

Taken together, these studies demonstrate direct and indirect roles for prolactin in bone physiology. However, from a clinical perspective, there remain many unknowns. First, whilst restoration of normal prolactin and gonadal function improves BMD, this is not always associated with complete normalization of BMD emphasizing the clinical importance of early treatment of hyperprolactinemia. Second, the effect of treatments for hyperprolactinemia on BMD and fracture risk has scarcely been examined (40,42). Third, most clinical evidence for a negative effect of hyperprolactinemia on bone is derived from patients with prolactinomas, despite medication-induced hyperprolactinemia representing the most frequent cause of non-physiological hyperprolactinemia (194), principally from neuroleptics and antipsychotic agents (195). However, there are some studies of medication-induced hyperprolactinemia implicating it in reduced bone density and increased fracture risk (196,197). Further research in this area is warranted to inform more robust clinical guidelines regarding the use of these medications from a bone health perspective (198). Finally, much data in humans has been derived from small,
heterogenous and cross-sectional or retrospective studies, which are likely to affect the
generalizability of the results. To improve the granularity of the data, larger, multicenter and
prospective studies are warranted, although the importance of PRL in overall bone physiology
remains unquestionable (Figure 1).

Progesterone

Most of the focus on gonadal reproductive hormones have involved estrogens and androgens.
However, progesterone is another critical gonadal hormone acting in tandem with estrogens as
a requisite hormone for maintaining fertility (199). Progesterone is produced in the gonads and
adrenal glands of both sexes. As with other gonadal steroids, progesterone is synthesized from
pregnenolone, which itself is derived from cholesterol (200).

*In Vitro Studies*

Within the bone microenvironment, both osteoblasts and osteoclasts express the progesterone
receptor (PR) (201,202). Furthermore, PR expression on osteoblasts can be stimulated by
estrogen, thus it is possible that some of the effects on bone physiology attributed to estrogen
may be mediated in part via enhanced progesterone signaling (201,203,204).

While estrogen’s role in decreasing bone resorption is an important factor for maintaining bone
health, there is evidence that progesterone contributes to bone formation through its actions on
osteoblastic cells. Low physiologic doses of progesterone increased osteoblastic production of
transforming growth factors (TGF)-β1, TGF-β2, and TGF-β3 mRNA (205) and bone-specific ALP
(205). Importantly, this effect was seen independently of pre- or co-treatment with estrogen
(206). Progesterone also regulates the function of metalloproteinases in cultures of human
osteoblast-like cells, which may have local effects on osteoblastogenesis or matrix remodeling
(205).
The effects of progesterone on osteoclasts and osteocytes have not been well-explored to date although PR has been found on osteoclasts (202) and chondrocytes (207).

*In Vivo* Non-Human Studies

To address whether PR signaling is requisite for normal bone growth and turnover, global knockout (PRKO) mice with deletions in both PR A and B isoforms have been studied. Histomorphometric and micro-CT analyses demonstrated normal longitudinal bone growth at the tibia (208,209), however total trabecular and cortical bone mass were increased at other skeletal sites such as the humerus and distal femur (209,210) suggesting that at certain sites, PR signaling attenuates the accumulation of cortical and trabecular bone mass during periods of rapid bone growth e.g. adolescence. In general, PRKO mice do not typically exhibit alterations in circulating levels of gonadal sex steroids, however loss of PR signaling is known to affect other upstream hormones including PRL and LH (199). Furthermore male PRKO mice display significantly lower FSH levels, whereas female PR knockout mice have higher inhibin A levels (210). Therefore, it is important to consider these additional hormonal consequences of PR deletion and their confounding impact on bone physiology.

*In Vivo* Human Studies

Post-menopausal estrogen deficiency is a major risk factor for osteoporosis, however it is now established that estrogen and progesterone work in tandem (206). Data from the Women's Health Initiative demonstrates that estrogen plus progesterone (compared to placebo) taken for an average of 5.6 years reduces the risk of fractures in post-menopausal women (Hazard Ratio 0.76 overall) (211). A more recent meta-analysis of over 1000 menopausal women reported that estrogen plus progesterone therapy resulted in superior increase in lumbar BMD compared to estrogen therapy alone (212), highlighting an additive action for progesterone.
During states of estrogen-deficiency such as amenorrhea, surgical and physiological menopause, low progesterone is almost indistinguishable temporally from low estrogen levels making it difficult to isolate its effects. However, progesterone deficiency also occurs silently in conditions of subclinical ovulatory disturbance (SOD) whereby ovulation is disturbed with shorter luteal phases but normal cycle length and preserved estrogen levels (213). SOD therefore provides a useful context for the study of progesterone effects on female bone. Pre-menopausal women with lower BMD exhibit significantly lowered progesterone levels despite regular cycles and frequently normal estrogen levels (213). Furthermore, studies of healthy pre-menopausal women have found levels of bone formation and resorption markers to change across the menstrual cycle with increased markers of bone formation and higher osteoblastic activity occurring during the (progesterone-rich) luteal phase. However, most of these studies have not been able to differentiate ovulatory from anovulatory cycles which limits the interpretation of their findings.

Interventional studies assessing the use of cyclical oral progesterone to provide luteal phase support in SOD have demonstrated some benefits in lumbar BMD after 1 year (cyclic medroxyprogesterone plus calcium +1.7% versus placebo plus calcium -0.7%) at doses that do not suppress endogenous estrogen production (214). While cyclical progesterone provides an interesting and potentially novel therapeutic option for women at risk of bone–related complications of SOD, caution should be applied given the highly heterogenous nature of patients presenting with SOD with variable energy expenditure, physical activity, and diet (213). Further interventional studies in this area may be useful in elucidating the role and therapeutic potential of progesterone in pre-menopausal bone health. These would be particularly useful given that by contrast to the above preclinical and interventional studies identifying positive effects, depot medroxyprogesterone acetate at contraceptive doses has consistently been shown to result in reversible BMD loss in longitudinal studies (215,216) and possible increased
fracture risk in observational studies (217,218). However, these findings likely represent the effects of supraphysiological progesterone levels to decrease levels of associated beneficial hormones (particularly estrogen).

The importance of progesterone in male physiology is less well-studied, however progesterone is known to suppress LH and testosterone in men and there is also evidence that progesterone therapy markedly increases BMD in a trial involving twenty-three steroid-dependent asthmatic men (219). However, further studies in this area are required to categorically isolate the effects of progesterone on bone physiology from associated hormonal confounders, potentially through the development of PR floxed mice with osteoblast specific deletion of the PR.

Relaxin

Relaxin (RLN) is a member of the insulin-like peptide superfamily (220), which consists of seven peptides of high structural similarity. During pregnancy, it is produced by the corpus luteum and placenta, hence it is traditionally recognized as a pregnancy hormone with significant roles in promoting cervical softening and elongation of the pubic symphysis to facilitate birth during the peripartum (221). There are seven established relaxin family peptides (RXFP), including relaxin (RLN)1, RLN2, RLN3, and insulin-like peptide (INSL)3, INSL4, INSL5 and INSL6 (222). Relaxin mediates its actions by binding to and activating the G-protein coupled RXFP receptors with RLN1, RLN2 and RLN3 the ligands for RXFP1, RXFP2 and RXFP3, respectively. In addition, INSL3 signals through RXFP2.

In Vitro Studies

Expression of RXFP1 mRNA and transcripts have been detected in primary cell cultures of human osteoclasts by RT-PCR and immunofluorescence analyses (223). In the same study, relaxin was shown to induce the differentiation of peripheral blood mononuclear cells (PMBC)
into mature osteoclasts, providing early evidence for the role of relaxin in osteoclastogenesis (223). Furthermore, relaxin itself is not produced by human osteoclasts (224), signifying that it acts on osteoclasts as a circulating endocrine factor, and not as an autocrine/paracrine mediator.

Relaxin induces the expression of classical stimulators of osteoclastogenesis implicated in the differentiation, survival and activation of osteoclasts, including RANK, NF-kB, NFATc1 and TRAP (224). Notably, relaxin induces the expression of the cysteine protease CTSK, a key enzyme produced by mature osteoclasts and involved in the resorption of the organic matrix of bone (224).

In addition to the data underscoring the role of relaxin as an osteoclast-activating factor to increases bone resorption, in vitro studies have examined its effect on osteoblastic cells. Treatment of the mouse calvarial osteoblast cell line MC3T3-E1 with recombinant human RLN1 increased ERK1/2 phosphorylation and increased the expression of Runx2 and ALP, whilst inhibiting OPG and RANKL expression (225). As a consequence of long-term exposure, relaxin inhibited collagen synthesis and enhanced matrix metalloproteinase activity (225).

Relaxin has been shown to synergistically augment BMP-2 induced osteoblast differentiation and bone formation in vitro, by upregulation of Runx2 expression and activity (226). In fact, a recent study examined the therapeutic application of relaxin as an enhancer of BMP-2 for bone regeneration, identifying that a combination of relaxin and BMP-2 significantly reduced the BMP-2 dose required to regenerate an equivalent amount of bone in rats (227).

RXFP2 mRNA expression and protein have been detected in osteosarcoma cell line MG-63 and also in primary osteoblast cell culture (228). Interestingly, while RXFP2 expression in human osteoblasts is ~20 lower than that of PTH receptor type 1, the level of expression is 20% higher than that seen in the testis (the primary site of INSL3 (which signals through RXFP2)
production) (228). Human osteoblasts respond in a dose- and time-dependent manner to INSL3 with respect to cAMP production and proliferation (228). Mechanistically, in cultured osteoblast progenitor cells, INSL3 treatment significantly induced ALP activity (229). Recent data also demonstrates that the INSL3/RXFP2 system acts on the MAPK cascade and stimulates the transcription of important genes of osteoblast maturation/differentiation and osteoclastogenesis (229).

Finally, expression of both Rxfp1 and Rxfp2 has been detected in the osteocytes lining the trabecular formations of developing mouse calvarial bones (225). However, unlike the more defined roles in osteoblast and osteoclast biology, no effect of relaxin on osteocytes has been reported to date (230).

**In Vivo Non-Human Studies**

Building on the *in vitro* data highlighting the role for INSL3 in bone physiology, RXFP2 deficient mice have been generated. Bone histomorphometric and micro-CT analyses at the lumbar and femoral sites revealed diminished bone mass and altered trabecular organization (228). Also, the mineralizing bone was reduced, resulting in a lower bone formation rate (228). In addition, the number of osteoclasts was maintained, but the osteoclast surface was reduced, signifying impaired osteoclast differentiation (228). Collectively, these findings suggest that the low bone mass in mutant mice is attributable to impaired bone formation, and a negative balance between bone formation and resorption.

A recent study generated gene knockouts for RLN1 by CRISPR-Cas9 technology (231). Newborn mutant pups exhibited small skeletal size at birth when compared with wild-type littermates, whereas adult mutant mice (12 weeks old) grew normally and showed normal bone density (231). This may imply that RLN1 is involved predominantly in prenatal bone development and has less of an effect on bone remodeling of postnatal bone.
Given its influence in stimulating local angiogenesis, vasculogenesis and osteogenesis, further animal experiments have investigated the potential therapeutic benefit for relaxin in accelerating bone fracture healing. Relaxin treatment did not accelerate closure of calvarial defects in mice, despite administering physiological to supraphysiological range doses to different mouse ages (3-4 and 13-14 months) and allowing for different investigational durations (232). On the other hand, relaxin appeared to enhance trabecular bone growth in an uninjured control bone (femur) (232). This may suggest an enhancing effect of relaxin on bone formation. Future studies are warranted using different experimental models of bone fracture and different species. However, to provide definitive data for the role of relaxin in bone physiology (Figure 1), studies involving osteoblast-specific deletion of the relaxin receptors expressed on osteoblasts (induced in adulthood) are necessary although the human study below provides promising (although not osteoblast-specific) data.

In Vivo Human Studies

Building on the data illustrating that disruption of RXFP2 signaling is associated with reduced bone mass in mice, a similar phenotype has been identified in humans. In a study of 25 young men (age 27-41 years) harboring a T222P mutation in the RXFP2 gene, 64% were found to have significantly reduced bone density, despite normal levels of testosterone and gonadal function and no other causes for reduced bone mass (228). Of significance, BMD levels of the femoral neck and lumbar spine revealed osteopenia in 10 subjects and osteoporosis in 6 subjects (228). Hence, this study provides an interesting link between the RXFP2/INSL3 hormonal system and bone mass. Future clinical studies may provide further insight on the phenotype in carriers of pathogenic variants in FXFP2 that may advance our understanding of the effects of FXFP2 on bone, such as by assessments of bone turnover markers and dynamic histomorphometry. Whether the relaxin-bone pathway can be manipulated for therapeutic purposes in humans remains to be explored.
Activin and Inhibin

Activin and inhibin are structurally related regulatory glycoprotein hormones with diametrically opposing biological effects on reproductive function. Activin, a member of the TFGβ superfamily, consists of three homodimer protein complexes linked by disulphide bridges: \( \beta_A\beta_A \) (activin A), \( \beta_A\beta_B \) (activin AB), and \( \beta_B\beta_B \) (activin B) (233). Inhibin, also belonging to the TFGβ superfamily, consists of two heterodimeric protein complexes: \( \alpha\beta_A \) (inhibin A) and \( \alpha\beta_B \) (inhibin B) (234). Inhibins are endogenous antagonists of activin-signaling governing pituitary FSH secretion and normal gonadal function.

Similar to the other TFGβ superfamily members, activin signals are transmitted through two types of transmembrane serine/threonine kinase receptors: activin type I receptors (i.e., ACVR1, ACVR1B and ACVR1C) and type II receptors (i.e., ACVR2A and ACVR2B). Initially, activins bind to a type II receptor, which results in the recruitment, phosphorylation and activation of type I receptor (235). Inhibin antagonizes activin-signaling through displacement of activin by binding to type II receptors through its \( \beta \)-subunits, without leading to phosphorylation of type I receptors (236,237).

Activins are produced in many organs, including the gonads, pituitary gland and placenta (238,239). In men, inhibin is secreted primarily from the Sertoli cells, whereas in women, it is produced more widely including the granulosa and theca cells of the ovary, as well as the pituitary gland and placenta (240). Outside of reproduction, both hormones have been shown to participate in a broad range of biological processes, including regulation of energy metabolism, inflammation and skeletal homeostasis (241). Regarding the latter, abnormal activin-signaling is implicated in certain skeletal disorders. In particular, mutations in the ACVR1 gene causes Fibrodysplasia Ossificans Progressiva, a rare disorder characterized by abnormal development.
of bone in skeletal muscle and connective tissue (termed heterotopic ossification) (242,243), further illustrating that activin-signaling plays a role in skeletal homeostasis.

In Vitro Studies

Using fetal-rat calvarial osteoblastic cultures, recombinant human activin A stimulated cell proliferation and both collagen and non-collagen protein synthesis (244). In primary murine bone marrow cultures, activin has a stimulatory effect on both osteoblasts and osteoclasts, whereas inhibin exhibited opposite effects (245). The stimulatory effect of locally produced activin on osteoblast and osteoclast differentiation does not however override the suppressive effects of inhibin produced by the gonads (245). Given that the suppressive effect of inhibin is maintained in the presence of activin or BMP (245), suggests the presence of a distinct inhibin-specific receptor. In addition, ACVR2A and ACVR2B have also been detected within murine cortical and trabecular bone osteocytes (246).

Consistent with observations in murine cells, both Inhibin A and Inhibin B suppress osteoblastogenesis and osteoclastogenesis in human skeletal and hematopoietic progenitor cells (247). Collectively, these data reveal that inhibins have direct negative effects on osteoblast and osteoclast differentiation and these effects are consistent in human and murine in vitro models.

In stark contrast to activins stimulatory effects in murine bone marrow cultures above (245), in several human osteoblast models, activin treatment dose-dependently inhibits matrix protein production and suppresses in vitro mineralization through autocrine signaling (248). This may reflect species differences or differences in the in vitro test system.
In Vivo Non-Human Studies

A number of studies demonstrate stimulatory effects of activin on bone formation in vivo. In neonatal rat calvaria, daily periosteal injections of activin promoted bone formation in a dose- and time-dependent manner (249). Furthermore, the effects of activin (administered intramuscularly three times a week for 12 weeks) in aged ovariectomized rats has been examined. In this study, activin markedly increased lumbar vertebral bone mass, mechanical strength, and compression strength of the vertebral body by 1.5-fold (250). Moreover, activin did not affect the urinary excretion of deoxypyridinoline, suggesting that activin enhanced bone formation rather than inhibited bone resorption (250). In keeping with these data, using a rat fibula fracture model, activin injected daily into the fracture site, enhanced callus formation in a dose-dependent manner (251). In fact, 3 weeks of treatment increased the mechanical strength of the healed fractured bone and histological analysis revealed that activin promoted bone formation (251). Collectively, these in vivo studies support a stimulatory role for activin on osteoblast activity.

In a transgenic mouse model engineered to overexpress liver-derived human InhA, the resultant continuous Inhibin A exposure led to increased total BMD, bone volume and increased biomechanical properties at the proximal tibia (252). In fact, Inhibin A also prevented gonadectomy-induced loss of BMD and bone volume and strength at both the spine and proximal tibia in male mice (252), suggesting in this model that inhibin may have an even larger influence than sex steroids in regulating bone mass. Also, Inhibin A increased mineral apposition rate, and serum OC levels in vivo and osteoblastogenesis in ex vivo cultures without affecting osteoclast number or activity (252). Taken together, these set of data suggest that the effects from Inhibin A are mediated through bone formation, rather than effects on osteoclasts.
Whether the increase in bone formation without overt change in osteoclast activity reflects uncoupling of bone remodelling remains to be investigated. These results seemingly contradict the aforementioned suppressive effects of Inhibin A in vitro (245,247), which may imply that the endocrine effects of continuous exposure in vivo may override the direct suppressive effects of inhibin A treatment on osteoblastogenesis in vitro. Further studies are required for clarification.

In vivo Human Studies

In a cross-sectional age-stratified study of 188 pre- and postmenopausal women, serum Inhibin A and Inhibin B levels were found to negatively correlate with bone formation (i.e. serum ALP) and to a lesser extent resorption markers (i.e. serum CTx and 24-hour urinary levels of pyridinoline and deoxypyridinoline of type I collagen) in premenopausal women and women of perimenopausal age (45-54 years) (247). In postmenopausal women Inhibin A (but not Inhibin B) negatively correlated with bone formation markers (247). Furthermore, using multivariate analyses, premenopausal serum Inhibin A levels exhibited stronger correlation with bone formation and resorption markers compared with either FSH or estradiol (247), suggesting circulating Inhibin A levels may be used as a clinical biomarker of high bone turnover and bone loss, before detectable changes in estrogen levels.

Consistent with these data, in a study of 87 regularly menstruating women aged 35-50 years, decreased Inhibin B levels were highly related to bone resorption, as indicated by increased excretion of urinary NTX (253). Furthermore, multivariate regression analysis revealed that serum Inhibin B levels were also an independent contributor of lumbar bone BMD (253), providing further evidence for the role of inhibin in bone physiology.

Taken together, the data above demonstrate positive bone effects of activin and a crucial role for inhibins in bone physiology, seemingly independent of other reproductive hormones (Figure 1). Indeed, these data suggest a role for the early decrease in inhibins as the result of early
ovarian failure, as a mechanism driving the observed perimenopausal accelerated bone turnover that precedes overt decreases in circulating estrogens. Based on these in vitro and in vivo findings, inhibin may therefore serve as a novel biomarker of bone loss in women with early follicular phase blood collection likely to be most useful (254) and warrants further study in larger cohorts. Moreover, unlike inhibin, activin has been less studied in human bone physiology, hence underscoring the need for further studies in humans. Particularly so given that activin-signaling has been proposed as a novel and emerging therapeutic target for osteoporosis (255–257), which requires further validation in human studies.

Conclusion

Skeletal homeostasis and the process of bone remodeling depends on the tightly regulated coupling of bone resorption and bone formation. Whilst it is traditionally held that gonadal sex steroids play a key role in bone physiology, recent advances have demonstrated an important role of other reproductive hormones in bone physiology that is independent of their role in reproduction, as discussed in this review (Figure 1). Our aim has been to present an overview of the literature into a single comprehensive assessment of the current state of the field. Kisspeptin, the master regulator of the HPG axis, influences osteoblast differentiation, with recent data indicating its role as a key mediator in a neuroskeletal circuit in the arcuate nucleus controlling bone formation. Whilst limited data exists regarding the direct effects of GnRH, emerging results from canine models reveals its participation in bone remodeling, potentially through interplay with the serotonergic system (although this is based on osteosarcoma cell lines which are not representative of normal bone cells). Whilst numerous observational studies in humans have produced results suggestive of direct FSH effects on bone, no human interventional study has detected a direct cause-and-effect relationship between FSH and bone turnover. LH is likely to have minimal influence on skeletal homeostasis with its effects
predominantly occurring via gonadal sex steroid changes. The direct influence of PRL to
stimulate bone turnover, with a greater involvement in bone resorption than formation, is now
well established and included in hyperprolactinemia-management decision making. Finally, the
roles for relaxin, activin and inhibin in bone physiology are increasingly recognized.

Crucially, many questions remain unanswered with a significant amount of knowledge gained
purely from in vitro models relying on cell lines or transformed cells. Despite these valuable
studies, very little is known regarding the effects of reproductive hormones on osteocyte biology.
Furthermore, the complex inter-relationship between the HPG hormones themselves makes
isolating their independent effects difficult experimentally, although successfully overcome by
several studies mentioned in the current review. Indeed, despite the multiplicity of in vitro
studies, clear mechanisms and pathway data remain to be fully defined and replicated. In an
effort to circumvent the inherent limitations associated with in vitro experiments, further in vivo
studies are necessary to establish the effects of reproductive hormones on the bone
microenvironment and downstream-signaling and to examine for sexual dimorphisms, as well as
additional studies in non-murine animals to clarify species differences. Notably, in vivo studies
frequently examine global knockout or transgenic mice resulting in long-term developmental
consequences and systemic sequelae, which may confound interpretation of the effects of
reproductive hormones on bone. Finally, it is also important to recognize that a significant
proportion of human data comes from observational studies, reporting simply associations and
are limited in numbers, therefore causality cannot be fully inferred.

An important way to address these fundamental problems will be the development of in vivo
models with inducible cell specific deletion of key receptors in bone cells to evaluate the roles of
reproductive hormones signaling pathways in bone. This has the ability to determine whether
certain reproductive hormones other than estrogen and testosterone have physiologically
important direct roles in the skeleton, with important therapeutic implications.
In summary, whilst the study of several reproductive hormones remains in its infancy and many mechanistic details remain to be identified, accumulating evidence highlights a wide range of reproductive hormones beyond estrogens and androgens as key components of the physiological endocrine inventory that regulates skeletal homeostasis. To this end, these data emphasize the existing and emerging therapeutic possibilities of related manipulations of the HPG system and its constituents for the prevention and treatment of metabolic bone diseases.
Abbreviations

ALP: Alkaline phosphatase
AP-1: Activator protein-1
AR: Androgen receptor
BMD: Bone mineral density
BMP: Bone morphogenetic proteins
BV/TV: Bone volume over total volume
Ctx: C-terminal telopeptide of type 1 collagen
Dlx5: Distal-less homeobox
ERα/β: Estrogen receptor alpha/beta
FSH: Follicle-stimulating hormone
GnRH: Gonadotropin-releasing hormone
hCG: Human chorionic gonadotropin
HPG: Hypothalamic-pituitary-gonadal
IGF-1: Insulin-like growth factor 1
KISS1: Kisspeptin
LH: Luteinizing hormone
M-CSF: Macrophage colony-stimulating factor
MAPKs: Mitogen-activated protein kinases
MSC: Mesenchymal stem cells
NF-κB: Nuclear factor-κB
NFATc1-4: Nuclear factor of activated T-cells, cytoplasmic 1-4
NTX: N-terminal telopeptide of type I collagen
OC: Osteocalcin
OPG: Osteoprotegerin
Osx: Osterix
PRL: Prolactin
PTH: Parathyroid hormone
RANKL: Receptor activator of nuclear factor-κB ligand
RLN: Relaxin
Runx2: Runt-related transcription factors 2
TRAF6: Tumour necrosis factor receptor-associated factor 6
TRAP: Tartrate-resistant acid phosphatase
TSH: Thyroid stimulating hormone
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Table and Figure Legends

Table 1: The Effects of Reproductive Disorders on Bone

Figure 1: Schematic of the Hypothalamic-Pituitary-Gonadal Reproductive Axis and Summary of the Direct Effects on Bone. Green shading denotes predominantly positive effect and red shading predominantly negative effects on skeletal homeostasis. Grey shading denotes no or uncertain overall effects on skeletal homeostasis.

ARC, hypothalamic arcuate nucleus; FSH, follicle stimulating hormone; FSHR, follicle stimulating hormone receptor; GnRH, gonadotropin-releasing hormone; GnRHR, gonadotropin-releasing hormone receptor; KISS1, kisspeptin; KISS1R, kisspeptin receptor; LH, luteinizing hormone; LHR, luteinizing hormone receptor; PR, progesterone receptor; PRLR, prolactin receptor.
| Reproductive Disorder                          | Effects on Bone                                                                                                                                 |
|-----------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------|
| Premature Ovarian Insufficiency (POI)         | - Low bone mass due to insufficient peak bone mass accrual (depending on onset) and increased bone remodelling (predominately bone resorption) secondary to estrogen deficiency (21)  
  - Bone loss greater in trabecular than cortical bone (22)  
  - Prevalence of osteoporosis of 8–14% (23)  
  - Almost 50% of patients have significantly reduced BMD within 1.5 years of POI diagnosis (24)  
  - 2-3% lower BMD at lumbar, femoral neck and hip compared to normally menstruating women (25) |
| Functional Hypothalamic Amenorrhea (FHA)      | - Bone loss related to the duration of amenorrhea and the degree of estrogen deficiency (26)  
  - Prevalence of low BMD in female athletes with FHA or oligomenorrhea estimated up to 15.9% (27)  
  - Average reduction in lumbar spine BMD of 15% by 3 years compared to normally menstruating women (26)  
  - Significant fracture risk, including stress fractures (28) |
| Congenital Hypogonadotropic Hypogonadism (CHH) | - Chronic gonadal steroid deficiency associated with reduced peak bone mass in early adulthood and accelerated bone loss (29)  
  - Prevalence of osteoporosis almost 45% of untreated young men with CHH (30)  
  - BMD improves during gonadal sex steroid replacement, especially in skeletally immature men (31), but does not fully reverse the skeletal abnormalities (32) |
| Pregnancy and Lactation-associated Osteoporosis (PLO) | - Mechanisms include negative calcium balance and lactational estrogen deficiency (33), along with additional hormonal changes predisposing to ligament laxity (34)  
  - Associated with fractures during late pregnancy or postpartum (35)  
  - Frequently manifests as severe back pain (36,37)  
  - Possible recurrence in subsequent pregnancies (38) |
| Hyperprolactinemia                            | - Decreased bone density due to direct and indirect (via hypogonadism) effects of prolactin on bone physiology (39)  
  - Associated with early alterations in bone turnover markers that precede BMD changes (40)  
  - Bone mass diminished predominantly in trabecular rather than cortical bone (41)  
  - Bone loss more marked when hyperprolactinemia develops at a younger age, which restricts peak bone mass acquisition (42)  
  - High risk of radiological vertebral fractures in men and women with PRL-secreting pituitary adenomas averaging 32-37% (43,44) |
Essential Points:

- Skeletal homeostasis is regulated by reproductive hormones and their fluctuations throughout life.
- Prevalent reproductive disorders, including Premature Ovarian Insufficiency, Hypothalamic Amenorrhea and Hyperprolactinemia, are common causes of low bone mass.
- Whilst it is traditionally held that gonadal sex steroids play a major role in skeletal homeostasis, research has advanced into the influence of other reproductive hormones on bone physiology.
Kisspeptin
- KISS1/KISS1R expressed in osteoblast and osteoclast lineage cell lines. Kisspeptin stimulates osteoblast precursor differentiation in mouse mesenchymal stem cells.
- Mediates a neuroskeletal circuit in the ARC modulating bone formation in mice.

GnRH
- GnRH and GnRHR expressed in canine osteosarcoma cell lines and to lesser extent in normal canine osteogenic progenitor cells. Peripheral GnRH signaling may participate directly in bone remodeling in osteosarcoma cell lines, potentially through RANKL and the serotonergic system.
- No data

Prolactin
- Osteoblasts (but not osteoclasts) express PRLR. PRL acts directly on bone to predominantly increase bone resorption (via osteoblast-osteoclast pathways).
- Stimulates intestinal calcium absorption during pregnancy and lactation, as well as direct and independent negative effects on bone remodeling.

FSH
- FSHR expressed in human osteoclasts and mesenchymal stem cells (but not in mature osteoblasts), with data supporting a direct role in osteoclastogenesis.
- Several (but not all) non-human experimental and human observational studies suggest negative bone effects. However, as yet no interventional human studies identifying direct bone effects.

LH
- LHR expressed on primary human osteoblasts and osteoblast-like cell lines.
- Effects on bone physiology predominantly mediated via gonadal sex steroids.

Testosterone
- PR expressed on osteoblasts and osteoclasts, with stimulatory effects on osteoclasts.
- Promotes bone formation in collaboration with estrogen.

Relaxin
- Receptor expressed by osteoclasts stimulating osteoclastogenesis. Positive effect on osteoclastogenesis.
- Positive effects on bone remodeling. Disrupted relaxin pathways reduces bone density.

Activin
- Stimulates osteoblastogenesis and osteoclastogenesis in primary murine bone marrow culture, but suppresses mineralization in human osteoblast preparation.
- Stimulatory effects on bone formation.

Inhibin
- Suppresses osteoblastogenesis and osteoclastogenesis in both primary murine bone marrow culture and human mesenchymal cells.
- Positive effects on bone formation in mice. Association with bone mass and turnover (inverse) in humans.