

Review

The Relationship Between Bone and Reproductive Hormones Beyond Estrogens and Androgens

Edouard G. Mills,¹ Lisa Yang,¹ Morten F. Nielsen,² Moustapha Kassem,^{2,3} Waljit S. Dhillon,^{1,4} and Alexander N. Comninou^{1,4,5}

¹Division of Diabetes, Endocrinology and Metabolism, Imperial College London, London, UK; ²Department of Endocrinology, University Hospital of Odense & Institute of Clinical Research, University of Southern Denmark, 5000 Odense C, Denmark; ³Faculty of Health and Medical Sciences, Department of Cellular and Molecular Medicine, University of Copenhagen, 2200 Copenhagen N, Denmark; ⁴Department of Endocrinology, Imperial College Healthcare NHS Trust, London, UK; and ⁵Endocrine Bone Unit, Imperial College Healthcare NHS Trust, London, UK

ORCID numbers: 0000-0003-1557-0869 (M. Kassem); 0000-0001-5950-4316 (W. S. Dhillon); 0000-0002-7104-2297 (A. N. Comninou).

Abbreviations: ALP, alkaline phosphatase; BMD, bone mineral density; BMI, body mass index; BMP, bone morphogenetic proteins; cAMP, 3',5'-cyclic adenosine 5'-monophosphate; Ctx, C-terminal telopeptide of type 1 collagen; Dlx5, distal-less homeobox; ER α / β , estrogen receptor alpha/beta; ERK, extracellularly regulated kinase; FSH, follicle-stimulating hormone; FSHR, follicle-stimulating hormone receptor; GnRH, gonadotropin-releasing hormone; GnRHR, gonadotropin-releasing hormone receptor; hCG, human chorionic gonadotropin; HPG, hypothalamic-pituitary-gonadal; IL, interleukin; INSL, insulin-like peptide; KISS1, kisspeptin gene; LH, luteinizing hormone; LHR, luteinizing hormone receptor; MAPKs, mitogen-activated protein kinases; M-CSF, macrophage colony-stimulating factor; mRNA, messenger RNA; NF- κ B, nuclear factor- κ B; 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; PR, progesterone receptor; PRKO mice, global knockout mice with deletions in PR A and B isoforms; PRL, prolactin; PRLR, prolactin receptor; PTH, parathyroid hormone; RANKL, receptor activator of nuclear factor- κ B ligand; rFSH, recombinant follicle-stimulating hormone; RLN, relaxin; RXFP, relaxin family peptides; Runx2, runt-related transcription factors 2; SOD, subclinical ovulatory disturbance; TGF, transforming growth factor; TSH, thyroid stimulating hormone.

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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

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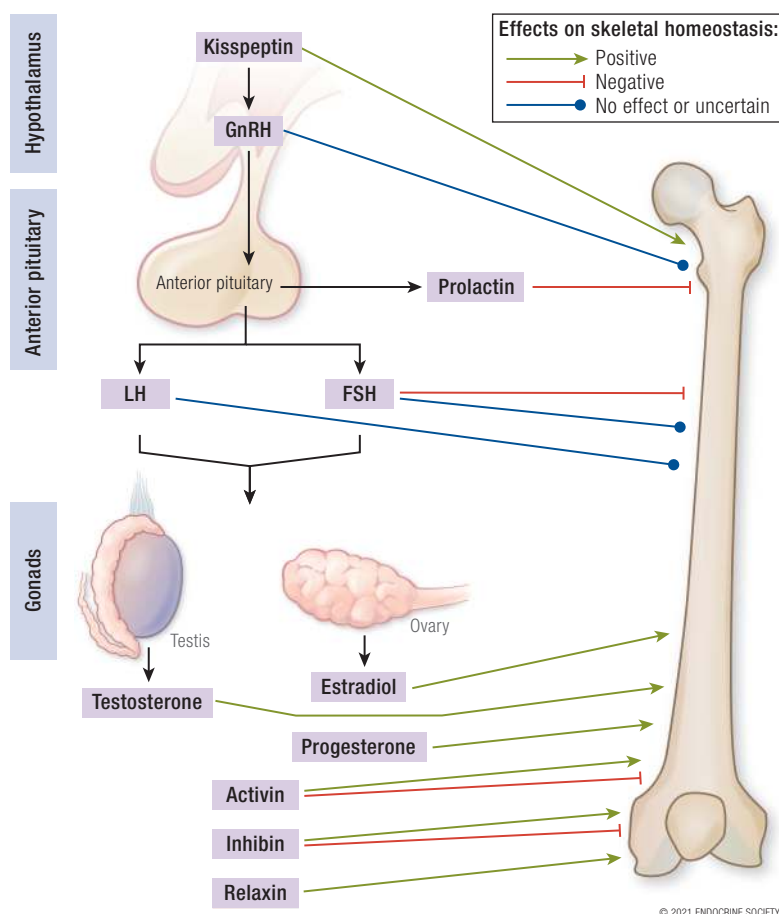
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between bone and the reproductive system. In this review, we provide a contemporary summary of the literature examining the relationship between bone biology and reproductive signals that extend beyond estrogens and androgens, and include kisspeptin, 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 aging population.

Key Words: bone, kisspeptin, GnRH, FSH, LH, prolactin, progesterone, inhibin, activin, relaxin

Graphical Abstract



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
- While 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

Skeletal homeostasis in mammals is tightly regulated by the process of bone remodeling, which 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 remodeling 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 both for bone (3) and reproduction (4). How bone remodeling 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 remodeling, including gastrointestinal hormones such as glucagon-like peptide 1, glucagon-like peptide 2, and glucose-dependent insulinotropic polypeptide (8, 9), as well as adipose-tissue factors such as adiponectin (10).

In skeletal diseases, bone remodeling 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 microarchitectural deterioration, and resultant susceptibility to fractures (11, 12). Postmenopausal bone loss is a central risk factor for developing osteoporosis (13, 14). The higher risk and prevalence of fractures results in disability, reduced quality of life, and increased mortality (15). Notably, the increasing incidence of osteoporosis (16) and health care costs associated with an aging 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, coexpression 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 (BMD), 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, estrogen 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, while the prevalence of fractures in women with PRL-secreting pituitary adenomas is high, this risk has been observed to be similar between amenorrheic and eugonadal premenopausal 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

Table 1. Effects of reproductive disorders on bone

Reproductive disorder	Effects on bone
Premature ovarian insufficiency (POI)	<ul style="list-style-type: none"> • Low bone mass due to insufficient peak bone mass accrual (depending on onset) and increased bone remodeling (predominately bone resorption) secondary to estrogen deficiency (21) • Bone loss greater in trabecular than cortical bone (22) • Prevalence of osteoporosis of 8% to 14% (23) • Almost 50% of patients have significantly reduced BMD within 1.5 years of POI diagnosis (24) • 2% to 3% lower BMD at lumbar, femoral neck, and hip compared to normally menstruating women (25)
Functional hypothalamic amenorrhea (FHA)	<ul style="list-style-type: none"> • Bone loss related to duration of amenorrhea and 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 y compared to normally menstruating women (26) • Significant fracture risk, including stress fractures (28)
Congenital hypogonadotropic hypogonadism (CHH)	<ul style="list-style-type: none"> • Chronic gonadal steroid deficiency associated with reduced peak bone mass in early adulthood and accelerated bone loss (29) • Prevalence of low BMD 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 skeletal abnormalities (32)
Pregnancy and lactation-associated osteoporosis	<ul style="list-style-type: none"> • 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) • Risk of recurrence in subsequent pregnancies (38)
Hyperprolactinemia	<ul style="list-style-type: none"> • 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% to 37% (43, 44)

Abbreviations: BMD, bone mineral density; PRL, prolactin.

as the urgent need to develop novel, safe, and effective therapies for low bone mass to prevent bone fractures in an aging population.

Materials and 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 “remodeling” 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 October 21, 2020.

The Hypothalamic-Pituitary-Gonadal 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” that first posited that the secretion of pituitary gonadotropins was controlled by chemical substances of hypothalamic origin released into the hypophyseal (pituitary) portal circulation (59). Consequently, this conceptual groundwork led to the isolation and sequencing of gonadotropin-releasing hormone

(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 (encoded by Kiss1/KISS1) administration acted as a potent stimulator of gonadotropin release in animals (64-67) and humans (68-70), whereas pretreatment with a GnRH antagonist abolished this stimulatory effect of kisspeptin (71).

In light of such fundamental discoveries, the historical understanding of the hormonal cascade that controls the HPG axis was revised (Figure 1): Kisspeptin sits at the apex of the reproductive axis and is secreted by hypothalamic KISS1 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 (luteinizing hormone [LH] and follicle-stimulating hormone [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 more than 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 epigenetic 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- κ B ligand (RANKL), osteoprotegerin (OPG), and dickkopf-1 (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 extracellularly regulated kinase (ERK) and protein kinase B (79). By comparison, RANKL binds to its receptor, RANK, leading to the recruitment of adaptor molecules, such as tumor necrosis factor receptor-associated factor 6, which subsequently leads to the activation of mitogen-activated protein kinases (MAPKs), and the transcription factors nuclear factor- κ B (NF- κ B) and activator protein-1 (80). Activated nuclear factor- κ B induces the nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), a major osteoclastogenesis regulator (80). Meanwhile, depending on their state of differentiation, osteoblasts (discussed later) 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). The commitment of mesenchymal stem cells toward 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, type 1 collagen and several noncollagen proteins such as osteocalcin (OC), osteopontin, and bone

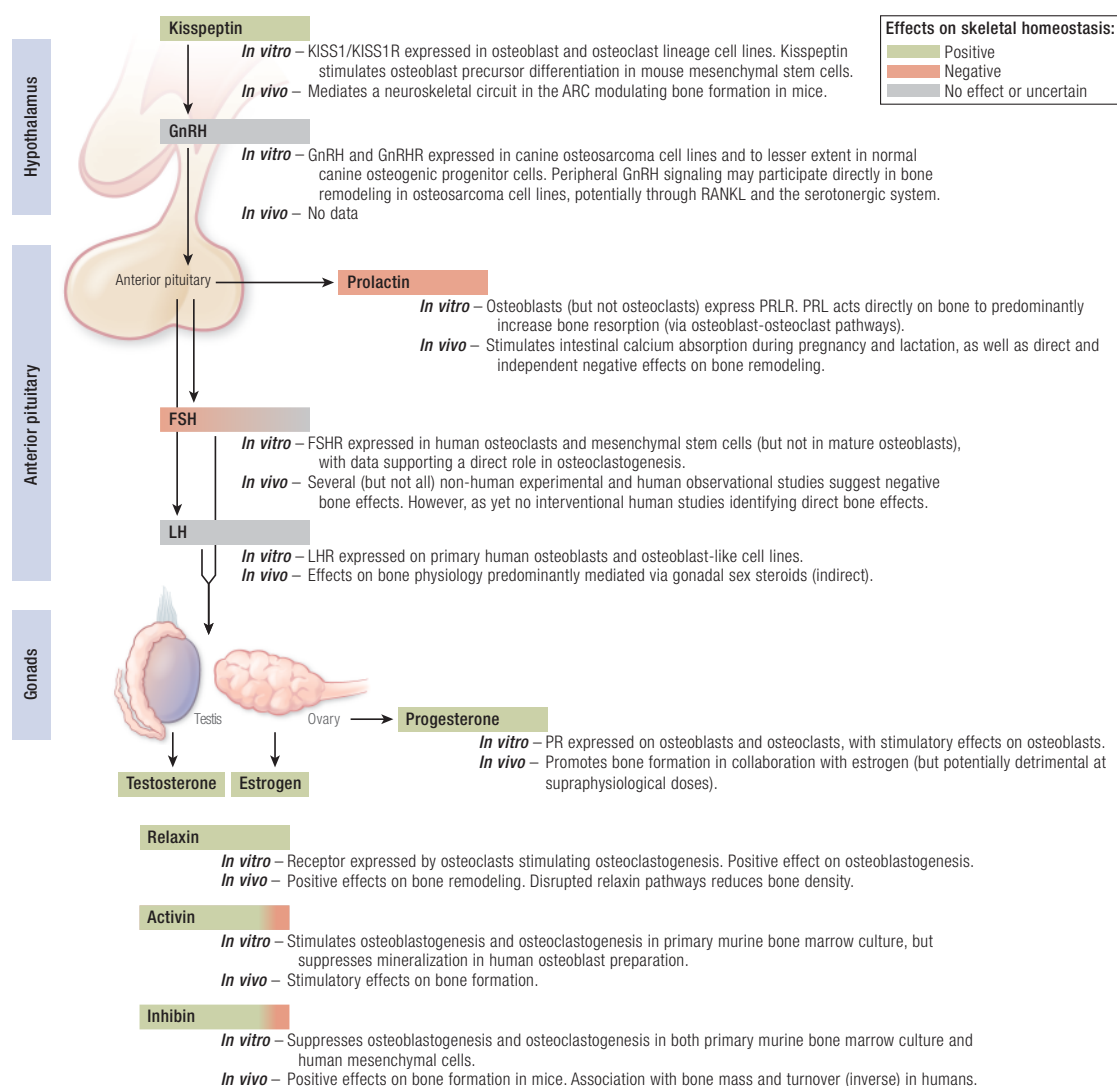


Figure 1. Schematic of the hypothalamic-pituitary-gonadal reproductive axis and summary of the direct effects on bone. Green shading denotes predominantly positive effect and red shading predominantly negative effects on skeletal homeostasis. Gray shading denotes no direct or uncertain overall effects 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 gene; KISS1R, kisspeptin receptor gene; LH, luteinizing hormone; LHR, luteinizing hormone receptor; PR, progesterone receptor; PRLR, prolactin receptor.

sialoprotein (84). Osteoblasts therefore mediate bone formation through the deposition of unmineralized osteoid matrix and its subsequent mineralization (85).

Each of these cell types is 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, and glucocorticoids (72, 86, 87), a wealth of emerging studies highlights the importance of reproductive hormones, as detailed in the present 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 nonhuman/human *in vivo* perspectives to ensure clarity and full appreciation of the important differences

between these methodologies and species, and the implications of their findings.

Kisspeptin

Kisspeptin refers to a family of structurally related endogenous peptides encoded by the human *KISS1* gene (nonhuman *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). While 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 behavior, 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 because of 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, 2 principal populations exist: (1) the infundibular nucleus, and (2) the rostral preoptic area (99, 100). In rodents, Kiss1 exists predominantly in 2 distinct populations: (1) the arcuate nucleus (equivalent to the human infundibular nucleus), and (2) the anteroventral periventricular nucleus (71, 101, 102). The detection of Kiss1 signaling in bone cell lines (103) may imply its putative role in bone physiology as detailed in the following section.

In Vitro Studies

KISS1 messenger RNA (mRNA) and protein are both 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 in which 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 (ie, 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 Kiss1 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 Kiss1r 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 Kiss1, 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 KISS1 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 KISS1 and its receptor are expressed in osteocytes and influence secretion of factors involved in remodeling (such as sclerostin) remains unknown and will no doubt be a focus of future investigation.

In Vivo Nonhuman Studies

A contemporary pivotal murine study using a combination of different genetic models and stereotaxic surgery demonstrated that deleting estrogen receptor alpha (ER α) 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 approximately 700% with an average 80% increase in bone volume over total volume (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 ER α 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 suggesting a possible therapeutic avenue for postmenopausal loss of bone mass. In addition, loss of ER α specifically in Kiss1-expressing arcuate cells recapitulated this bone phenotype, defining central Kiss1 signaling as a key mediator in ER-neuroskeletal circuit (113). Importantly, arcuate Kiss1 neurons are well established as major neurons involved in coordinating energy states with reproduction (114). Therefore, it is interesting to speculate that these Kiss1 neurons are part of a wider system that controls energy-demanding bone remodeling to maintain reproduction.

Based on the earlier described 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 investigations 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 reveal new therapeutic avenues.

Gonadotropin-Releasing Hormone

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 7-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 2 gonadotropins (LH and FSH) (116).

In many vertebrates, 3 forms of GnRH (GnRH I, II, and III) have been identified, although only 2 exist in reptiles, birds, and mammals (117). Correspondingly, 3 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 in the following section.

In Vitro Studies

While data from primary cell culture are lacking, a recent study provided potential evidence for direct effects of GnRH on osteoblast-like cells. Both in 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 tumor 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 4- to 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 KISS1) treatment increased both COS proliferation and the expression of the bone remodeling ligand RANKL (but not OPG expression), effects that were blocked by treatment with a GnRHR inhibitor (105). GnRH and kisspeptin-10 treatment also increased the expression of the serotonin receptor htr2a 2- to 8-fold (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 proliferative effects in these osteosarcoma cells. These results, although based on nonhealthy (osteosarcoma) cell lines, provide an interesting insight into a possible role for GnRH on bone remodeling, potentially through interplay with the serotonergic system (see Figure 1). Owing to the short half-life of GnRH of 2 to 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 hypophyseal-portal circulation and has a very short half-life as mentioned earlier, circulating levels cannot currently be analyzed in peripheral blood, making clinical studies challenging. Conversely, a plethora of studies have examined the effect of GnRH agonists on 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 a 2% to 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% to 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 5 years after a prostate cancer diagnosis, 19.4% of those who received androgen-deprivation therapy (orchidectomy or GnRH agonist) experienced a fracture (most commonly the femoral neck, rib, spine, and hand), compared with 12.6% of those who did not receive androgen-deprivation therapy ($P < .001$) (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 drawing definitive conclusions regarding the effects of GnRH agonists vs antagonists on bone.

In addition to prostate cancer, GnRH agonists are also commonly employed as a treatment strategy to suppress ovarian function in the management both of endometriosis (129) and premenopausal/perimenopausal 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 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 a combination with tamoxifen resulted in a lesser decline of -1.4% (132). It is notable that only partial recovery from bone loss was observed on cessation of goserelin treatment alone at 1 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 the 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 also 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 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 most likely result predominantly from suppressing the release of subsequent downstream reproductive hormones, which have more established effects on bone.

Follicle-Stimulating Hormone

FSH is synthesized by gonadotrope cells in the anterior 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 predominantly regulates testicular development and spermatogenesis (136).

FSH is a glycoprotein dimer consisting of an alpha (α) and beta (β) subunit. Whereas 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 Gs α /3',5'-cyclic adenosine 5'-monophosphate (cAMP)/protein kinase 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 the placenta, umbilical cord vessels, uterus, liver, and bone (139). Hence, interest in 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, 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 3 established osteoclastogenic pathways by enhancing the phosphorylation of Erk1/2, I κ B α , and protein kinase B 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 (ie, similar to circulating follicular-phase FSH levels of women during reproductive years) or 100 mIU/mL (ie, FSH levels after menopause) had no significant influence on RANK expression (141). By comparison, at 50 IU/mL (ie, 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 described earlier, FSH has also been implicated in modulating the activity of several proinflammatory cytokines involved in regulating osteoclastic bone resorption. Murine bone marrow cultures exposed to recombinant FSH stimulated tumor necrosis factor α 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 interleukin (IL)-1 β (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 Nonhuman 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 ovariectomized 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), FSH β -deficient homozygous female mice did not lose bone, with both areal and volumetric BMD increased (140). Comparatively, FSH β -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 known that LH and testosterone both become elevated in FSH β and FSHR null mice (144-146), which makes a definite conclusion about the direct role of FSH from these experiments somewhat uncertain. Therefore, to fully establish that complete loss of FSH signaling in FSH β and FSHR null mice protects from bone loss (despite severe hypogonadism), ovariectomy in the mutant mice (thus eliminating gonadal sex steroids including 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 include

genetic or chemical approaches 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).

In contrast to the aforementioned studies, further experimental data reveal that a direct role for FSH in bone remains unclear. By age 3 months, 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 potentially 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 that results in increasing circulating FSH levels with age (151). In this study, increased FSH induced bone formation and increased bone mass, an effect that 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, while FSH and LH were observed to modulate bone loss, changes in estrogen had a more powerful influence (152). Taken together, while 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 so studies in male mice would be useful 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 these findings.

In Vivo Human Studies

Numerous observational studies report an association between increasing serum FSH levels and bone loss. The multisite, longitudinal Study of Women's Health Across the Nation examined 2375 premenopausal and early perimenopausal women of African American, White, Japanese, and Chinese background (153). In these premenopausal 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 body mass index [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 premenopausal 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. A total of 289 postmenopausal women were genotyped for the single-nucleotide polymorphism 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 that 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 were 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 eumenorrhoeic women younger than 40 years, amenorrhoeic women had lower lumbar BMD (although no difference in femoral neck BMD) than eumenorrhoeic women (157). The amenorrhoeic women were then separated into 2 groups according to their FSH levels: hypergonadotropic amenorrhea (ie, FSH > 40 IU/L) and hypogonadotropic amenorrhea (ie, 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 vs 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 workup there was no difference in BMD between the 2 groups, despite a significantly higher median FSH value (9.8 vs 3.7 IU/L) (158). Importantly, total testosterone and estradiol were similar between the 2 groups at follow-up (158). Indeed, neither the baseline nor follow-up FSH levels exhibited a 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 OC (159). Interestingly, FSH was inversely correlated with serum levels of soluble RANKL in both cohorts of men, an effect that remained significant after adjustment for relevant nonhormonal confounders (age, body fat percentage, and smoking in the idiopathic infertility cohort, compared with age adjustment only in the Klinefelter syndrome cohort) and serum estradiol (159). Critically, while 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 postmenopausal 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 suggesting 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 the 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 both in premenopausal and postmenopausal 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 (see 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) studies in women and men with comprehensive additional bone assessments could be illuminating. Ultimately, although 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

The glycoprotein hormone LH is a heterodimer consisting of a noncovalently 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 7-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, the established skeletal changes seen in puberty, pregnancy, and menopause (169) (ie, during periods of amplified levels of LH/hCG), may functionally

imply that LH and hCG may participate directly in bone physiology as discussed in the following sections.

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 reverse transcriptase-polymerase chain reaction (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 was not reported.

By contrast, other investigators have observed that human osteoblasts treated with a urine-derived formulation of hCG resulted in osteoblast proliferation as indicated by elevated ALP activity and increased matrix metalloproteinase 2 expression (170). In fact, although hCG alone was capable of stimulating an increase in ALP activity, cotreatment with hCG and calcitriol resulted in a 5-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 (170). Taken together, these data do not robustly support that hCG influences human osteoblasts, given there was no response when nonurine-derived hCG was used. Moreover, whether LH/hCG directly influences osteoclast or osteocyte biology remains to be answered.

In Vivo Nonhuman Studies

To explore the putative role of LH/hCG on the skeleton in vivo, an LH receptor null mutant mouse model and a murine transgenic model overexpressing both hCG subunits (hCG $\alpha\beta$ +) have been generated (167). Ablation of LHR resulted in a 43% reduction in femoral BMD by age 5 months, and histomorphometric analysis revealed reduction in cancellous bone volume, trabecular width, and number (167). Interestingly, 6-month-old male hCG $\alpha\beta$ +/ mice had comparable BMD to wild-types, and female hCG $\alpha\beta$ +/ mice exhibited approximately 30% increases both in 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 increase in BMD was a direct effect of raised serum hCG, or an indirect ovarian effect, female hCG $\alpha\beta$ + and wild-type mice were bilaterally ovariectomized at age 3 weeks, which resulted in 36% and 33% reductions in femoral and tibial BMD, respectively (167). Collectively, these data support 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 because of 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 transforming growth factor (TGF)- β 2 in perimenopausal women (171). Additionally, in postmenopausal women, LH levels also showed a positive correlation with OPG levels, but not with TGF- β 2 (171). These observations provide potential mechanistic insight into bone physiology during perimenopausal and postmenopausal periods (ie, at times of amplified LH levels with concentrations up to 10 times those found premenopause) 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, while the influence of FSH was approximately 7 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 years) from the United States, 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 postmenopausal Turkish women with low vs normal femoral and lumbar BMD (175). Given the previous studies, it is possible that the latter 2 studies failed to detect an association because of 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 that did not however 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, 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 LH levels and BMD has been inconsistent across different populations. It is important to recognize that because 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 (see Figure 1).

Prolactin

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 other corresponding changes in circulating estrogens and progesterone amongst others. Notably, these physiological states are recognized to induce a maternal bone-resorptive state 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 PRL 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 D₃ (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 approximately 2- to 3-fold in the presence of 200 to 500 ng/mL PRL (187). Interestingly, only higher PRL concentrations of 500 ng/mL (ie, comparable to the average suckling-induced PRL surge) upregulated TNF- α and IL-1 by approximately 3-fold and approximately 2-fold, respectively, whereas M-CSF and IL-6 mRNA expressions were unaffected by 100 to 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 approximately 2-fold after exposure to 300 ng/mL PRL (187). This latter finding suggests that PRL can also facilitate osteoblast-osteoclast communication through direct cell-cell contact using the ephrin system *in vitro* (187). Moreover, the 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 the 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, resulting in increased bone resorption rather than formation through osteoblast-related pathways.

In Vivo Nonhuman Studies

In keeping with *in vitro* models, *in vivo* animal experiments reveal a prominent role for PRL in calcium and bone homeostasis. Studies in rats during pregnancy and lactation 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 the 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

intestinal 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 the 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 because of the 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 because of 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 (ie, 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 both from *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 aforementioned data suggest direct effects, the presence of other reproductive hormone-dependent effects is plausible. Future studies employing techniques such as cell-specific gene targeting (eg, for the

osteoblastic *PRLR*) 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, 4 patients were found to have normal BMD both at 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 become 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 (42). 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 have demonstrated that vertebral BMD decreases by 20% to 30%, while forearm BMD decreases by 2.5% to 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 the 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

nonfracture 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 insulin-like growth factor 1 levels 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 to 12 months of medical treatment, the improvement in BMD both at 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 PRL in bone physiology. However, from a clinical perspective, there remain many unknowns. First, although restoration of normal PRL 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 limited data as yet (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 nonphysiological 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, many data in humans have been derived from small, heterogeneous, 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 (see Figure 1).

Progesterone

Most of the focus on gonadal reproductive hormones involves 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, osteoblasts and osteoclasts both 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 TGF- β 1, TGF- β 2, and TGF- β 3 mRNA (205) and bone-specific ALP (205). Importantly, this effect was seen independently of pretreatment or cotreatment 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 identified on osteoclasts (202) and chondrocytes (207).

In Vivo Nonhuman Studies

To address whether PR signaling is requisite for normal bone growth and turnover, global knockout (PRKO) mice with deletions both in PR A and B isoforms have been studied. Histomorphometric and microcomputed tomography 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, such as 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

Postmenopausal 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 demonstrate that estrogen plus progesterone (compared to placebo) taken for an average of 5.6 years reduces the risk of fractures in postmenopausal women (hazard ratio, 0.76 overall) (211). A more recent meta-analysis of randomized controlled trials of more than 1000 menopausal women reported that estrogen plus progesterone therapy resulted in a 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, and 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 (214). Premenopausal women with lower BMD exhibit significantly lower progesterone levels despite regular cycles and frequently normal estrogen levels (213). Furthermore, studies of healthy premenopausal women have found levels of bone formation and resorption markers 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% vs placebo plus calcium -0.7%) at doses that do not suppress endogenous estrogen production (215). 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 heterogeneous 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 premenopausal bone health. These would be particularly useful given that by contrast to the aforementioned 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 (216, 217) and possible increased fracture risk in observational studies (218, 219). However, these findings likely represent the effects of supraphysiological progestin levels that 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 although there is also evidence that progesterone therapy markedly increased BMD in a trial involving 23 steroid-dependent asthmatic men (220). 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 (221), which consists of 7 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 (222). There are 7 established relaxin family peptides (RXFP), including relaxin (RLN)1, RLN2, RLN3, and insulin-like peptide (INSL)3, INSL4, INSL5, and INSL6 (223). 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 reverse transcriptase-polymerase chain reaction and immunofluorescence analyses (224). In the same study, relaxin was shown to induce the differentiation of peripheral blood mononuclear cells into mature osteoclasts, providing

early evidence for the role of relaxin in osteoclastogenesis (224). Furthermore, relaxin itself is not produced by human osteoclasts (225), 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- κ B, NFATc1, and tartrate-resistant acid phosphatase (225). 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 (225).

In addition to the data underscoring the role of relaxin as an osteoclast-activating factor increasing 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 while inhibiting OPG and RANKL expression (226). As a consequence of long-term exposure, relaxin inhibited collagen synthesis and enhanced matrix metalloproteinase activity (226).

Relaxin has been shown to synergistically augment BMP-2–induced osteoblast differentiation and bone formation *in vitro* by upregulation of Runx2 expression and activity (227). 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 (228).

RXFP2 mRNA expression and protein have been detected in the osteosarcoma cell line MG-63 and also in primary osteoblast cell culture (229). Interestingly, while RXFP2 expression in human osteoblasts is approximately 20 times lower than that of PTH receptor type 1, the level of expression in these cells is 20% higher than that seen in the testis (the primary site of INSL3 [which signals through RXFP2] production) (229). Human osteoblasts respond in a dose- and time-dependent manner to INSL3 with respect to cAMP production and proliferation (229). Mechanistically, in cultured osteoblast progenitor cells, INSL3 treatment significantly induced ALP activity (230). Recent data also demonstrate that the INSL3/RXFP2 system acts on the MAPK cascade and stimulates the transcription of important genes of osteoblast maturation/differentiation and osteoclastogenesis (230).

Finally, expression both of Rxfp1 and Rxfp2 has been detected in the osteocytes lining the trabecular formations of developing mouse calvarial bones (226). However, unlike the more defined roles in osteoblast and osteoclast biology,

no effect of relaxin on osteocytes has been reported to date (231).

In Vivo Nonhuman Studies

Building on the *in vitro* data highlighting the role for INSL3 in bone physiology, RXFP2-deficient mice have been generated. Bone histomorphometric and microcomputed tomography analyses at the lumbar and femoral sites revealed diminished bone mass and altered trabecular organization (229). Furthermore, the mineralizing bone was reduced, resulting in a lower bone formation rate (229). In addition, the number of osteoclasts was maintained, but the osteoclast surface was reduced, signifying impaired osteoclast differentiation (229). 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 (232). Newborn mutant pups exhibited small skeletal size at birth when compared with wild-type littermates, whereas adult mutant mice (age 12 weeks) grew normally and showed normal bone density (232). This may imply that RLN1 is involved predominantly in prenatal bone development and has less of an effect on bone remodeling in postnatal bone.

Given its influence in stimulating local angiogenesis, vasculogenesis, and osteogenesis, further animal experiments have investigated the potential therapeutic benefit of 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 (233). On the other hand, relaxin appeared to enhance trabecular bone growth in an uninjured control bone (femur) (233). 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 (see Figure 1), studies involving osteoblast-specific deletion of the relaxin receptor expressed on osteoblasts (induced in adulthood) are necessary, although the human study discussed later 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 (229). Of significance, BMD levels of the femoral neck and lumbar spine revealed osteopenia in 10 participants and osteoporosis in 6 participants (229). Hence, this study provides an interesting link between the *RXFP2/INSL3* hormonal system and bone mass. Future clinical studies may provide further insight into the phenotype in carriers of pathogenic variants in *FXFP2* that may advance our understanding of the effects of this signaling-system 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 TGF β superfamily, consists of 3 homodimer protein complexes linked by disulfide bridges: $\beta_A\beta_A$ (activin A), $\beta_A\beta_B$ (activin AB), and $\beta_B\beta_B$ (activin B) (234). Inhibin, also belonging to the TGF β superfamily, consists of 2 heterodimeric protein complexes: $\alpha\beta_A$ (inhibin A) and $\alpha\beta_B$ (inhibin B) (235). Inhibins are endogenous antagonists of activin-signaling governing pituitary FSH secretion and normal gonadal function.

Similar to the other TGF β superfamily members, activin signals are transmitted through 2 types of transmembrane serine/threonine kinase receptors: activin type I receptors (ie, ACVR1, ACVR1B, and ACVR1C) and type II receptors (ie, ACVR2A and ACVR2B). Initially, activins bind to a type II receptor, which results in the recruitment, phosphorylation, and activation of type I receptor (236). Inhibin antagonizes activin signaling through displacement of activin by binding to type II receptors through its β subunits, without leading to phosphorylation of type I receptors (237, 238).

Activins are produced in many organs, including the gonads, pituitary gland, and placenta (239, 240). In men, inhibin is secreted primarily from the Sertoli cells, whereas in women, it is produced more widely including by the granulosa and theca cells of the ovary, as well as the pituitary gland and placenta (241). 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 (242). 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*) (243, 244), 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 noncollagen protein synthesis (245). In primary murine bone marrow cultures, activin has a stimulatory effect both on osteoblasts and osteoclasts, whereas inhibin exhibited opposite effects (246). 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 (246). That the suppressive effect of inhibin is maintained in the presence of activin or BMP (246) 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 (247).

Consistent with observations in murine cells, both inhibin A and inhibin B suppress osteoblastogenesis and osteoclastogenesis in human skeletal and hematopoietic progenitor cells (248). 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 activin stimulatory effects in murine bone marrow cultures discussed above (246), in several human osteoblast models, activin treatment dose-dependently inhibits matrix protein production and suppresses in vitro mineralization through autocrine signaling (249). This may reflect species differences or differences in the in vitro test system.

In Vivo Nonhuman 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 (250). Furthermore, the effects of activin (administered intramuscularly 3 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 1.5-fold (251). Moreover, activin did not affect the urinary excretion of deoxypyridinoline, suggesting that activin enhanced bone formation rather than inhibited bone resorption

(251). 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 (252). In fact 3 weeks of treatment increased the mechanical strength of the healed fractured bone, and histological analysis revealed that activin promoted bone formation (252). 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 inhibin A, the resultant continuous inhibin A exposure led to increased total BMD, bone volume, and increased biomechanical properties at the proximal tibia (253). In fact, inhibin A also prevented gonadectomy-induced loss of BMD and bone volume and strength both at the spine and proximal tibia in male mice (253), 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 (253). Taken together, these 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 the uncoupling of bone remodeling remains to be investigated. These results seemingly contradict the aforementioned suppressive effects of inhibin A *in vitro* (246, 248), 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 premenopausal and postmenopausal women, serum inhibin A and inhibin B levels were found to negatively correlate with bone formation (ie, serum ALP) and to a lesser extent resorption markers (ie, 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) (248). In postmenopausal women, inhibin A (but not inhibin B) negatively correlated with bone formation markers (248). Furthermore, using multivariate analyses, premenopausal serum inhibin A levels exhibited a stronger correlation with bone formation and resorption markers compared with either FSH or estradiol (248), 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 to 50 years, decreased inhibin B levels were highly related to bone resorption, as indicated by increased excretion of urinary NTX (254). Furthermore, multivariate regression analysis revealed that serum inhibin B levels were also an independent contributor of lumbar bone BMD (254), providing further evidence for the role of inhibin in bone physiology.

Taken together, these data demonstrate the positive bone effects of activin and a crucial role for inhibins in bone physiology, seemingly independent of other reproductive hormones (see 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 (255) 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 (256–258) that 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. While it is traditionally accepted that gonadal sex steroids play the 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 (see 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. Although limited data exist regarding the direct effects of GnRH, emerging results from canine models reveal its participation in bone remodeling, potentially through interplay with the serotonergic system (although this is based on osteosarcoma cell lines that are not representative of normal bone cells). While 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 RLN, 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 interrelationship between the HPG hormones themselves makes isolating their independent effects difficult experimentally, although these have been successfully overcome by several studies mentioned in the present 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 nonmurine 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 that 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 that simply report 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, while 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.

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Additional Information

Correspondence: Alexander N. Comninou, BSc, MBBS, MRCP, PhD, Endocrine Bone Unit, Section of Endocrinology and Investigative Medicine, Division of Diabetes, Endocrinology and Metabolism, Department of Metabolism, Digestion and Reproduction, Imperial College London, London, W12 0NN, UK. Email: a.comninou@imperial.ac.uk.

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