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# The Role of GH/IGF-I-Mediated Mechanisms in Sex Differences in Cortical Bone Size in Mice

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## Abstract

Cortical bone dimensions are important determinants of bone strength. Gender differences in cortical bone size caused by greater periosteal expansion in males than in females during the pubertal growth spurt are well established both in humans and in experimental animal models. However, the mechanism by which gender influences cortical bone size is still a matter of investigation. The role of androgens and estrogen in pubertal bone growth has been examined in human disorders as well as animal models, such as gonadectomized or sex steroid receptor knockout mice. Based on the findings that growth hormone (GH) and insulin-like growth factor I (IGF-I) are major regulators of postnatal skeletal growth, we and others have predicted that sex hormones interact with the GH/IGF-I axis to regulate cortical bone size. However, studies conflict as to whether estrogen and androgens impact cortical bone size through the canonical pathway, through GH without IGF-I mediation, through IGF-I without GH stimulation, or independent of GH/IGF-I. We review recent data on the impact of sex steroids and components of the GH/IGF axis on sexual dimorphism in bone size. While the GH/IGF-I axis is a major player in regulating peak bone size, the relative contribution of GH/IGF-dependent mechanisms to sex differences in cortical bone size remains to be established.

### Keywords

Bone formation and resorption; IGF-I; Growth hormone; Androgen; Estrogen

## The GH/IGF-I Axis in Pubertal Bone Growth

The major cause of osteoporosis-related fracture is compromised bone strength. The key factors that determine bone strength include bone density, matrix composition, microarchitecture, and overall bone geometry and size [1]. The size of the bone is a



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particularly important contributor to overall bone strength [2,3]. However, the mechanisms that regulate bone size during various growth periods are not well understood. Puberty is associated with accelerated periosteal expansion in both genders. Boys have greater periosteal expansion than girls; therefore, bone diameter is greater in boys. Boys exhibit less endocortical apposition than girls, so the marrow diameter is smaller in girls [4]. However, these findings may depend on bone site and methodology [5]. In addition to an increase in circumference, longitudinal growth velocity peaks at 9 cm/year in girls and 10.3 cm/year in boys during puberty [6]. The later and longer pubertal growth spurt in boys results in a 10% difference in height and a 25% difference in peak bone mass in males compared to females [7].

The growth hormone (GH)/insulin-like growth factor I (IGF-I) axis is critical to the longitudinal pubertal growth spurt. The peak in longitudinal growth velocity is correlated to peak concentrations of GH [6,8]. Prior to puberty, GH release is similar in boys and girls. During puberty, the pulse amplitude of GH rises, and this occurs earlier in girls than in boys. IGF-I, similarly, rises earlier in puberty in girls. Levels of GH and IGF-I then drop after Tanner stage 5 in both sexes [6,9,10]. Thus, it has been generally assumed that sex steroids contribute to the higher GH/IGF-I action during the pubertal growth period.

Although the IGF systems in humans and mice differ in their relative concentration of IGF-I and IGF-II [11], the importance of the overall GH/IGF axis to bone growth has been demonstrated in animals with alterations of this pathway. Femoral bone mineral content in GH-deficient rats was reduced by 49% in males and 37% in females; femoral length was reduced 6.3% in males and 5.1% in females [12]. Knockout mouse data also clearly demonstrate a role of the GH/IGF-I axis in bone growth [13-15]. The loss of circulating IGF-I in various models has been shown to impact both bone length and width [16], as does the loss of locally produced IGF-I in osteoblasts [14] and chondrocytes [17]. However, these studies measure appendicular growth in different ways (tibia vs. femur; bone mineral content, bone length, dry bone weight, bone volume, periosteal circumference, etc.). Here, we review data on the role of GH/IGF-I in appendicular cortical bone size rather than longitudinal growth. Recently, we analyzed several knockout mouse models with the same methodology in order to directly compare cortical bone size phenotypes.

The cross-sectional area (CSA) of cortical bone in the femur was measured by pQCT [15] in order to directly compare four mouse models which are critical to elucidating this pathway: (1) GH-deficient *lit/lit* mice, which have a mutation in GH-releasing hormone receptor [18]; (2) total IGF-I knockout mice, in which the IGF-I gene is disrupted in every cell in the body [19]; (3) conditional IGF-I knockout mice, in which the IGF-I gene is disrupted only in type I collagen-producing osteoblasts [14]; and (4) total IGF-II knockout mice, in which the IGF-II gene is disrupted in every cell in the body [20]. Femoral size of GH-deficient *lit/lit* mice was decreased by 37% compared to wild-type controls, and femurs of total IGF-I knockout mice were smaller by 67% vs. wild-type, suggesting that IGF-I has some GH-independent contribution to cortical bone size. An ablation of total IGF-I was more severe than locally produced IGF-I. Conditional IGF-I knockout mice, lacking the IGF-I gene in collagen-producing osteoblasts, displayed a 31% decrease in femoral CSA, approximately half of the decrease seen in mice completely lacking IGF-I. Finally, total loss of IGF-II resulted in a 21% decrease in bone size, showing that IGF-II is less critical compared to IGF-I in regulating bone size. These data suggest that GH, IGF-I, and IGF-II may act via both common and unique mechanisms to regulate cortical bone size during growth.

To assess the gain in femoral CSA during the pubertal growth spurt, bone size was measured at 23 and 31 days of age [21]. Wild-type controls for each strain showed a >20% increase in femoral CSA during this time period. IGF-II knockout mice had a similar increase as wild-

type mice, while GH-deficient *lit/lit* mice and conditional IGF-I knockout mice had approximately 10% less of a pubertal increase than controls. Total IGF-I knockout mice had the smallest pubertal increase in CSA (15% less than controls), showing its primary influence on periosteal expansion. The interpretation of genetically engineered models is always complicated by the compensatory mechanisms that may exist in knockout mice. However, it is clear that the GH/IGF-I pathway is essential for periosteal expansion during the pubertal growth period. It is unknown, however, whether differences in GH/IGF-I in the two sexes are responsible for bone sexual dimorphism.

## Sex Steroids and Pubertal Bone Growth

Traditionally, it was assumed that androgens were growth-promoting and estrogen was growth-inhibiting in bone. This notion is supported by decreased growth in orchidectomized [12,22-24] and increased growth in ovariectomized [24-26] rats and mice, as well as reversal of these effects by replacement of the missing sex hormones. The effects of gonadectomy are smaller than the effects seen in GH/IGF-I-altered mice. For example, orchidectomized mice have a 9% decrease in femoral CSA [23]; orchidectomized rats have a 5% decrease in tibial CSA [26]. The results of ovariectomy are even smaller in scale. Femoral circumference increased by 3% in ovariectomized mice [25] and tibial CSA, by 1% in rats [26]. However, the differences seen in gonadectomized mice generally support the idea of androgens being growth-promoting and estrogen being growth-inhibiting (Table 1).

Conversely, human pubertal data have demonstrated that estrogen is, in fact, growth-promoting and that a substantial portion of testosterone's effect is mediated through its aromatization to estrogen. Estrogen receptor blockers such as tamoxifen prevent testosterone's therapeutic growth effect; nonaromatizable androgens are less effective in boys with delayed puberty. Men with a mutation in estrogen receptor alpha or in the aromatase gene do not have a pubertal growth spurt, while androgen-insensitive individuals do [6,9,27-30]. Estrogen supplementation stimulated growth in children with delayed puberty, and an aromatase inhibitor was effective in treating the over-growth of precocious puberty [31]. Although information from studies which examined solely longitudinal growth during puberty can be applied only to regulation of the growth plate cartilage, some of these assess cortical bone size as well [32]. If a substantial portion of androgen's effect on periosteal expansion is mediated via its aromatization to estrogen, then it is unclear why estrogen-induced periosteal expansion is greater in males than in females during the pubertal growth spurt.

It is also difficult to reconcile human clinical data involving aromatization of androgen to estrogen with the results of gonadectomy experiments in animals. It may be that estrogen plays different roles in humans (stimulatory) and rodents (inhibitory). However, a few studies support the role of estrogen as stimulatory in rats and mice. Vanderschueren et al. [33] compared the effects of orchidectomy versus an aromatase inhibitor in rats. Orchidectomy decreased multiple bone parameters, including an 11% reduction in CSA; the aromatase inhibitor decreased CSA by 15%. Similarly, Venken et al. [23] demonstrated that orchidectomized mice recovered femoral CSA by treatment with testosterone but not with nonaromatizable DHT or testosterone plus an aromatase inhibitor. Although the latter two treatments did have some effectiveness, this shows that maximal stimulatory effect is dependent on testosterone's aromatization to estrogen. Additionally, the overexpression of aromatase in osteoblasts in transgenic mice is stimulatory to bone mass (9.5% increase in tibial cortical CSA and decreased endosteal circumference) [34].

If estrogen is indeed stimulatory to pubertal bone growth in rodents, we are left with several unexplained ovariectomy experiments that caused increased bone growth [25,26]. Fritton et

al. [35] hypothesized that the response to gonadectomy depends on the state of the GH/IGF axis; however, this effect may be age-dependent, and puberty was complete prior to ovariectomy in their mice.

Multiple factors can also complicate the interpretation of gonadectomy experiments. For one, gonadectomy involves the removal of more than just the sex steroids. For example, inhibin A, a member of the TGF-$\beta$ superfamily, is derived from the gonad and has been shown to increase bone mass [36]. Another issue is that the gonads are not the only source of sex steroids. Adrenal androgens and, by aromatization, estrogens begin to rise at ages 6–8 in humans (adrenarche) [37], and adrenal androgens have been positively correlated to radial bone density, area, and bone mineral content [38]. About half of testosterone, one-third to one-half of estrone, and 4% of estradiol in women in the follicular phase is derived from the adrenal gland. Approximately 1–2% of circulating testosterone, one-third to one-half of estrone, and 10% of estradiol in men is adrenally derived [39]. More recently, Labrie et al. [40] have shown that as much as 40% of androgens in the prostate are independent of testicular testosterone. Adrenarche can occur in the absence of gonadarche and vice versa [39]. Although it has been traditionally assumed that rodents do not undergo adrenarche, Pignatelli et al. [41] demonstrated a transient rise in sex steroid synthesis and expression of 17a-hydroxylase in the rat prior to gonadarche. Adrenal tumors have also been shown to form and secrete sex steroids following gonadectomy in male CE/J mice [42]. The effect of adrenalectomy on periosteal growth has not been measured, but its effect on longitudinal growth (through regulation of cartilage signaling) has been demonstrated in rats [43].

Additionally, adrenal androgens can be converted to estrogens. Both humans and rodents express aromatase in the gonads and brain, and humans additionally have peripheral aromatase expression in the bone and adipose tissue [44]. A recent study demonstrated expression of aromatase in the gonadal fat pad of the male mouse but no other adipose site in the male or female [45]. Thus, the issue of whether factors produced by adrenal glands contribute to gender differences in cortical bone size remains uncertain.

Sex steroid receptor knockout mice avoid the complications of gonadectomy (Table 1). The decreased femoral cortical CSA (13% reduction) in androgen receptor knockout (ARKO) mice supports the stimulatory role of androgens without a dependence on aromatization to estrogen [23]. While the disruption of estrogen receptor $\beta$ (ER$\beta$) increased bone size in female, but not male, mice [46-48], the loss of estrogen receptor $\alpha$ (ER$\alpha$) decreased femoral CSA by 14% [47] and periosteal perimeter by 9% [49] in males. This evidence of both androgens and estrogen being stimulatory to periosteal bone growth is supported by recent work by Callewaert et al. [50]. Several measured parameters show decreases in AR and *ER$\alpha$* knockout mice, and the effects were additive in the combined knockout. For example, tibial cortical area was decreased by 17% in ARKO mice, 7.2% in ER$\alpha$KO mice, and 24.3% in double ARKO/ER$\alpha$KO animals.

However, even sex steroid receptor knockout models can be challenging to interpret. Kousteni et al. [51] showed that both E2 and DHT could inhibit apoptosis in osteoblasts and osteoclasts in culture. Using Hela cells (with no sex steroid receptors), they showed that both hormones had the antiapoptotic effect regardless of which receptor was transfected (ER$\alpha$, ER$\beta$, or AR). Thus, the assumption that the deletion of a receptor abolishes its ligand's effect may be inaccurate. Additionally, these knockout models were generated on various genetic backgrounds. The influence of mouse strain on bone size has been established [52,53]. The genetic background onto which a gene-targeted allele is placed can cause considerable variation in the phenotype of the genetically engineered mouse.

A hypothesized model which integrates the results of these mouse experiments is presented in Fig. 1. We propose that the smaller cortical bone size in females may be due to an inhibitory effect of estrogen via ER$\beta$ and the larger cortical bone size in males is due to the stimulatory effect of androgens and estrogen via AR and ER$\alpha$.

## Interaction of Sex Steroids with the GH/IGF-I Axis

Despite the uncertainty about the exact role of sex steroids in pubertal bone growth, many have proposed that the mechanism by which sex steroids have influence is via the GH/IGF-I axis. Both androgens and estrogen can stimulate GH secretion [30] and the increased irregularity of GH secretion characteristic of puberty [54], with testosterone's effect being dependent on aromatization to estrogen. In males, the androgen-induced increase in GH is correlated with an increase in IGF-I. In females, the estrogen-induced increase in GH is sometimes accompanied by a decrease in IGF-I. This phenomenon seems to be dosage-dependent, with higher doses being inhibitory of IGF-I release, and can be dependent on route of administration [29,55]. This leads to a hypothesis that in early puberty the sex steroids (including low levels of estrogen) are stimulatory to periosteal bone growth through the GH/IGF-I axis, while in late puberty high levels of estrogen in females are inhibitory to bone formation [32]. Although the rise of GH and IGF-I concentrations correlates to increased androgen and estrogen production, sex steroid levels remain high into adulthood while GH and IGF-I return to prepubertal levels [6,9,10]. Females, both adults and children, are less responsive to GH than males [8,56,57]. The mechanisms by which androgens and estrogens interact with GH to modulate transcriptional regulation of GH response genes remain to be established.

Even data from experiments that conflict about estrogen's role in increasing or decreasing pubertal bone growth can support its interaction with GH/IGF-I. The decreased appendicular bone growth in ER$\alpha$KO mice is accompanied by lower IGF-I levels (Table 1) [46,47]; this fits with a model of estrogen stimulating increased GH and IGF-I and growth in early puberty. In a model of estrogen being inhibitory to this pathway, Govoni et al. [25] found that ovariectomy of 3-week-old mice increased IGF-I and periosteal expansion (Table 1) and that supplementation with supraphysiological levels of estrogen partially reduced bone parameters and IGF-I levels to normal. Additionally, the ovariectomy-induced increase in appendicular size in rats was shown to be dependent on GH; female GH-deficient rats did not show the same effect of gonadectomy [12,22].

However, Venken et al. [58] demonstrated that estrogen can upregulate IGF-I and skeletal growth in the absence of the GH receptor, casting doubt on the role of the traditional GH/IGF-I axis. Questions about testosterone's interaction with this axis are even more substantial.

Traditionally, the correlation between rises in testosterone, GH, and IGF-I was thought to reflect a causal cascade [59]. However, orchidectomy at 6 weeks of age reduces axial and appendicular bone size in both GH-intact and GH-deficient rats, showing that testosterone's effect is additive and independent of GH [12,22]. Orchidectomy at 3 weeks of age reduces periosteal bone formation in mice without a decrease in serum IGF-I (Table 1) [23]. Androgens can increase periosteal bone growth even in the absence of the GH receptor and with no increase in IGF-I. Even in wild-type orchidectomized mice, androgen supplementation restores bone growth without an IGF-I increase [60].

Additionally, GH and IGF-I may have effects that are not dependent on each other. Prepubertal growth inhibition was more severe in IGF-I knockout mice than in GH-deficient mice, showing that IGF-I has effects on bone that are independent of GH. During puberty, both GH-dependent and GH-independent mechanisms come into play [15]. Mice deficient in

liver IGF-I display high GH, unchanged IGF-I, and increased cortical bone after ovariectomy, suggesting a direct role of GH in bone growth [35]. GH supplementation increases bone formation in IGF-I knockout mice [13], again demonstrating separate roles for these hormones.

Many of these studies examine serum levels of IGF-I. These results may not reveal differences in the local pool of available IGF, and perhaps IGF binding proteins mediate sex differences via local effects. Mice that overexpress IGFBP-5 display an equal reduction in femoral periosteal circumference in males and females, but endosteal circumference is decreased more in females than males [61]. IGFBP-2 null mice exhibit an increase in cortical thickness (and periosteal circumference) in the femur in females but a decrease in males [62]. Femoral cortical area in mice lacking the acid labile subunit is more severely reduced in males than females [63]. More research is needed to understand the mechanism behind these gender differences and whether sex steroids can directly up- or downregulate the expression of IGFBPs.

Therefore, the role of the GH/IGF-I axis in sexual dimorphism of bone growth, in whole or in part, is still under question. If steroid hormones influence the GH/IGF-I axis, then abolishing one or more parts of this axis should abolish bone size differences between males and females. Surprisingly, data from our laboratory show that sexual dimorphism in femoral CSA at 8 weeks of age was not impacted by the loss of any components of the GH/IGF-I pathway [21]. GH-deficient *lit/lit* mice, total IGF-I knockout mice, type I collagen–producing osteoblast-specific IGF-I knockout mice, total IGF-II knockout mice, and all corresponding wild-type controls retained sexual dimorphism, with CSAs being 9–17% larger in males than females. However, a recent report by Callewaert et al. [24] contradicts this finding, with GH receptor knockout mice exhibiting no differences between males and females in CSA of the tibia. There are several potential explanations for this discrepancy. First, our study using *lit/lit* mice with GH-releasing hormone receptor mutation may not be completely GH-deficient. It is possible that residual GH may be adequate for the gender effects on cortical bone size compared to GH receptor knockout mice used by Callewaert et al. Second, the *lit/lit* mice used in our study were generated on an inbred C57Bl/6J genetic background, while the GH receptor knockout mice used by Callewaert et al. represented a mixed (129Ola/Balb/c) genetic background. Third, CSA of femur vs. tibia was evaluated in *lit/lit* and GH receptor knockout mice, respectively. Obviously, more research is needed to clarify this issue. However, it is possible that a GH/IGF-independent mechanism is at least partially responsible for regulating gender differences in bone size in mice (Fig. 1).

## Possible Non-GH/IGF-I-Mediated Mechanisms for Sexual Dimorphism

If sex steroids cause sexual dimorphism in bone at least partly independently of the GH/IGF-I pathway, other mechanisms must be explored. First, sex hormone effects on periosteal bone cells may be direct. In this regard, steroid receptors (AR, ER$\alpha$, and ER$\beta$) are expressed in osteoblasts and osteocytes, and there are some conflicting data in osteoclasts [reviewed in 64]. Examples of direct effects include a decrease in osteoclast formation and function due to testosterone [65] and the direct promotion of apoptosis of osteoclasts by estrogen [66]. Androgens have been shown to both inhibit and stimulate osteoblasts, depending on the length of the treatment [67]. Mice that overexpress AR in osteoblasts have increased periosteal growth [68]. Multiple studies demonstrate that sex steroids can affect bone cells in culture, bypassing GH/IGF-I [69,70]. Accordingly, our data rule out a role for locally produced IGF-I in mediating gender effects on bone size since the sexual dimorphism in bone size was unaffected in osteoblast-specific conditional IGF-I knockout mice.

Second, sex hormones may influence periosteal cells by influencing autocrine/paracrine production of one or more cytokines. For example, both estrogen and testosterone have been shown to influence production of RANKL and OPG in osteoblasts [71]. Estrogen can also decrease proinflammatory cytokines that affect the preosteoclast pool [7]. Androgens may induce proliferation and differentiation of osteoblasts through the TGF-$\beta$ or IL-6 pathway, although results are conflicting [64] and location-specific [72]; androgens can also suppress RANKL-induced formation of osteoclasts [64].

Third, sex steroid effects on periosteal expansion may be through mechanotransduction. Estrogen receptors have been shown to mediate the response to strain both in vitro [73,74] and in vivo [75,76], although some of this effect may depend on paracrine IGF-I and IGF-II [77,78]. Additionally, it is assumed that androgens may have an indirect effect on bone by stimulating muscle growth, which increases mechanical loading [79]. The lack of a bone phenotype in the myocyte-specific ARKO mouse calls this into question however [80]. Thus, the exact mechanism by which sex hormones exert different effects on periosteal bone expansion remains to be established.

## Conclusions

Puberty is a transitional period between childhood and adulthood when substantial periosteal expansion and bone mass accrual are attained. The pubertal growth spurt is influenced by a number of factors, of which the GH/IGF-I axis is considered to be the major regulator. Because sex hormones stimulate GH production and interact with the IGF-I signaling pathway, a role for the GH/IGF-I axis in mediating gender differences in bone size has long been assumed. However, based on the aforementioned discussion, it is imperative that GH/IGF-I-independent mechanisms also be investigated for their role in sex differences in bone size. It is assumed that the findings from mouse models are applicable to humans, but one cannot rule out the possibility that the relative contribution of the GH/IGF axis to mediation of the effects of estrogen and androgen may be different in mice vs. humans. Since bone size is an important determinant of bone strength, defining the precise mechanism by which gender influences bone size is a prime area of future research.

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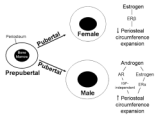


**Fig. 1.**

Hypothesized model of the mechanism by which estrogen and androgens regulate periosteal expansion during the pubertal growth spurt. Bone size increases during puberty but to a greater extent in males than in females. In female mice, a surge in estrogen during puberty may inhibit periosteal expansion by acting via ER*β* as bone size is increased in mice with a disruption of ER*β*. In male mice, androgens may regulate periosteal expansion directly by acting on bone cells via the androgen receptor (*AR*). In addition, estrogen produced by aromatases can act on periosteal expansion via ER*α* since bone size is decreased in male mice with a disruption of ER*α*. Androgen's effect on periosteal expansion may in part be mediated via a GH/IGF-I-independent mechanism based on the data from knockout mouse models (Table 1)

**Table 1**

Research on the influence of sex steroids on appendicular cortical bone size and IGF-I in mice

| Model | Sex steroid effect | Sex | Bone size | IGF-I level | References |
|---|---|---|---|---|---|
| ER$\alpha$KO | ↓ Estrogen effect via $\alpha$ receptor | Male | ↓ | ↓ | [47,49,50] |
| ER$\alpha$KO | ↓ Estrogen effect via $\alpha$ receptor | Female | ↔ | ↓ | [46] |
| BERKO | ↓ Estrogen effect via $\beta$ receptor | Female | ↑ | ↑ | [46] |
| BERKO | ↓ Estrogen effect via $\beta$ receptor | Male | ↔ | ↔ | [47] |
| DERKO | ↓ Estrogen effect via $\alpha$ and $\beta$ receptors | Female | ↔ | ↔ | [46] |
| DERKO | ↓ Estrogen effect via $\alpha$ and $\beta$ receptors | Male | ↓ | ↓ | [47] |
| Ovariectomy | ↓ Estrogen effect | Female | ↑ | ↑ | [25] |
| ARKO | ↓ Androgen effect | Male | ↓ | ↔ | [23,50] |
| Orchidectomy | ↓ Androgen effect | Male | ↓ | ↔ | [23] |
| Double ARKO/ER$\alpha$KO | ↓ Androgen effect and ↓ estrogen effect via $\alpha$ receptor | Male | ↓ | ↓ | [50] |