Regulation of male fertility by the bone-derived hormone osteocalcin

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Abstract

Traditionally, bone has been viewed as a relatively static tissue only fulfilling mechanical and scaffolding function. In the past decade however, this classical view of the bone has considerably evolved towards a more complex picture. It is now clear that the skeleton is not only a recipient for hormonal input but it is also an endocrine organ itself. Through the secretion of an osteoblast-derived molecule, osteocalcin, the skeleton regulates glucose homeostasis and male reproductive functions. When undercarboxylated, osteocalcin acts following its binding to a G-coupled receptor, GPRC6A, on pancreatic β cells to increase insulin secretion, on muscle and white adipose tissue to promote glucose homeostasis and on Leydig cells of the testis to favor testosterone biosynthesis. More recently, it was also shown that osteocalcin acts via a pancreas-bone-testis axis that regulates, independently of and in parallel to the hypothalamus-pituitary-testis axis, male reproductive functions by promoting testosterone biosynthesis. Lastly, in trying to expand the biological relevance of osteocalcin from mouse to human, it was shown that GPRC6A is a potential new susceptibility locus for primary testicular failure in humans. Altogether, these results shed new light on the importance of the endocrine role of the skeleton and also provide credence to the search for additional endocrine functions of this organ.

The classical view of the bone physiology

The skeleton is essential for locomotion and is defined primarily by its mechanical and scaffolding properties. This is critical for vertebrates to maintain a constant bone mass with high bone quality and excellent biomechanical properties. This is achieved by the ability of the bone to constantly renew itself through a mechanism called bone remodeling 1,2. Bone remodeling is a biphasic process including the destruction of the preexisting bone (bone resorption mediated by the osteoclasts), followed by a second phase of de novo formation of the bone, (bone formation mediated by the osteoblasts) 2-4. Importantly, these two phases not only occur sequentially but also in a balanced manner to keep a constant bone mass throughout life. A mis-regulation of this balance leads to diseases, the most frequent being osteoporosis, which is caused by an increase of bone resorption in comparison to bone formation 1,2,4-7. The regulation of bone (re)modeling is complex and involves mechanical stimuli, locally produced factors and many hormones. For instance, sex steroid hormones...
play a crucial role during the bone growth spurts of puberty, and for maintenance of bone mass\textsuperscript{3,7-11}.

**The novel dimension to the bone physiology**

Bone remodeling occurs throughout life in dozens of location in the skeleton, which is also one of the organs covering the largest surface in our body. Both the cellular events it entails and the surface covered by the skeleton suggest that this physiological process is costly energy-wise. Clinical observations support, fully, this view of bone (re)modeling. Specifically, the absence of food intake, as in anorectic children causes a near-total arrest of growth and low bone mass in adulthood\textsuperscript{12-14}. Moreover, and unrelated to food intake, it has been known for a long time that the growth and integrity of both the female and the male skeleton are influenced by sex steroid hormones. The biological importance of this regulation is best exemplified by the fact that gonadal failure triggers bone loss in both genders and leads to osteoporosis in post-menopausal women\textsuperscript{15,16}. Taken together, this view of bone (re)modeling and these clinical observations suggest that there may be a coordinated regulation of bone mass or growth, energy metabolism and reproduction\textsuperscript{1,17}.

Many genetic-based studies have shown that this hypothesis is true in both rodents and humans. The skeleton secretes at least two hormones. First, fibroblast growth factor 23 (FGF 23) regulating mineral metabolism through its control of the phosphate homeostasis that is intimately linked to bone health\textsuperscript{18,19}. Second, an osteoblast-specific secreted protein, osteocalcin, when undercarboxylated, acts as a multifunctional hormone. Osteocalcin acts on pancreatic $\beta$ cells to increase their proliferation and insulin secretion\textsuperscript{17,20,21}. It also promotes glucose homeostasis by acting in various tissues, such as muscle, liver and fat\textsuperscript{17,20,21} (Figure 1).

Subsequently, two groups working independently showed that mice lacking the insulin receptor in osteoblasts ($InsR^{osb-/-}$ mice) were glucose intolerant and insulin insensitive when fed on normal chow; that is, they were a phenocopy of the osteocalcin-gene-deficient mice\textsuperscript{22-24}. Because mice lacking the insulin receptor in skeletal muscle or white adipose tissue do not display glucose intolerance when fed a normal diet\textsuperscript{25,26}, insulin must act in additional tissues to achieve glucose homeostasis. The fact that bone is such a tissue legitimizes the notion that this tissue is necessary for glucose homeostasis. In addition, $InsR^{osb-/-}$ mice had significantly less biologically active (undercarboxylated) osteocalcin in their sera, revealing that insulin signaling in osteoblasts is a determinant of osteocalcin bioactivity\textsuperscript{25,26}. In a manner that is both elegant and economical, insulin uses the interplay between osteoblasts and osteoclasts for that purpose. Specifically, insulin inhibits the expression in osteoblasts of the gene encoding osteoprotegerin ($Opg$)\textsuperscript{23}, which hampers osteoclast differentiation. In other words, insulin signaling in osteoblasts favors bone resorption, a process that occurs at pH 4.5\textsuperscript{27}. Acidic pH is the only mechanism known to achieve decarboxylation of proteins\textsuperscript{28}, therefore, bone resorption decarboxylates and activates osteocalcin\textsuperscript{23} (Figure 2). Thus, in a feed forward loop, insulin signaling in osteoblasts promotes its own secretion by activating osteocalcin. Furthermore, mice and humans in which bone resorption is genetically impaired show a decrease in the undercarboxylated form of osteocalcin, resulting in glucose intolerance\textsuperscript{23} (Figure 2).

The discovery of the hormonal functions of osteocalcin raised multiple questions with great biological and medical importance. Chief among them was to elucidate the signaling events triggered by this hormone in target cells. A prerequisite to address this question was the identification of a specific receptor for osteocalcin. Because most hormones have several functions, the next question was whether this was the case for osteocalcin.
Osteocalcin favors testosterone production by the Leydig cells of the testis

The well-known regulation of bone remodeling by gonads \(^5,6,9,10,29\) suggested also that bone may in turn, through its endocrine capacity, affect reproductive functions in one or both genders. Verifying this hypothesis would further enhance the emerging concept that bone, energy metabolism and reproduction are coordinately regulated.

To test the validity of this hypothesis, a cell biology approach showed that osteoblasts secrete factor(s) that could markedly increase testosterone production by testis explants and primary Leydig cells \(^30\). The specificity of this function was verified in three ways: First, the supernatant of osteoblast cultures could not enhance sex steroid production in ovary explants. Second, they could not induce estradiol production by Leydig cells. Third, no other mesenchymal cell type shared this ability with osteoblasts \(^30\). This novel role of osteoblasts was verified recently in vivo \(^31\).

The fact that osteocalcin is a bone-derived hormone and that \(\text{Osteocalcin}^{-/-}\) mice aforementioned bred poorly suggested that ability of osteoblast culture supernatant could be due to osteocalcin. Testing this hypothesis relied on the use of a gain of function model for osteocalcin (\(\text{Esp}^{-/-}\) mice) and a loss-of-function one (\(\text{Osteocalcin}^{-/-}\) mice) \(^21\). \(\text{Osteocalcin}\)-deficient mice showed a decrease in testes, epididymides and seminal vesicles weights whereas the weight of these organs was increased in \(\text{Esp}\)-deficient mice. The spermogram of male \(\text{osteocalcin}\)-deficient mice showed a 50% decrease in sperm count, while the one of male \(\text{Esp}\)-deficient mice showed a 30% increase in this parameter. Leydig cell maturation appears to be halted in absence of \(\text{Osteocalcin}\) \(^30\). These features suggested that osteocalcin might favor testosterone synthesis. Again, this was verified by the simple but powerful co-culture assay, and then in vivo \(^30\). Indeed, circulating testosterone levels are low in \(\text{Osteocalcin}^{-/-}\) and high in \(\text{Esp}^{-/-}\) mice. Consistent with the fact that the supernatant of osteoblast cultures do not affect estradiol production, osteocalcin also does not affect the expression of genes encoding the aromatase enzymes, and estrogen levels are within the normal range in \(\text{Esp}^{-/-}\) and \(\text{Osteocalcin}^{-/-}\) mice \(^30\). To formally establish that osteocalcin regulates testosterone production as a bone-derived hormone and not as a testis-secreted growth factor, mice lacking \(\text{Osteocalcin}\) only in osteoblasts were generated. Male \(\text{Osteocalcin}_{\text{osb}}^{-/-}\) mice had the same testosterone production defect as the classical \(\text{Osteocalcin}^{-/-}\) mice, while deletion of \(\text{Osteocalcin}\) in Leydig cells did not affect male fertility \(^30\). Taken together these experiments established that osteocalcin is a bone-derived hormone favoring fertility in male mice by promoting Leydig cell maturation and testosterone production (Figure 1 and 3). In other words it verified that for at least one gender there is an endocrine regulator of reproduction by the skeleton. It also illustrates the existence of major differences in the regulation of fertility between male and female mice. With hindsight, these observations were both surprising and expected. They were surprising because bone is not classically seen as an endocrine organ much less one regulating reproduction. They were surprising also because of the absence of regulation of fertility in females. On the other hand, they were expected because the feedback rule that applies to most endocrine regulations suggested that given the fact that sex steroid hormones regulate bone mass in both genders, such a feedback regulation might exist. In broader terms, the existence of this function increases the importance of osteocalcin as a hormone.

Osteocalcin reproductive function is mediated by a G-coupled receptor, \(\text{Gprc6a}\)

In the molecular era, the identification of a novel hormone begs immediately the question of its mechanism of action. A prerequisite to answering this question is to characterize a
receptor to which this hormone would bind specifically on its target cells. In the case of osteocalcin, this was achieved through a two-step strategy taking advantage of the fact that osteocalcin regulates fertility in males but not in females. In the first step it was asked what the signal transduction pathway affected by osteocalcin is in two target cells, the β-cell of the pancreas and the Leydig cell of the testis. This approach identified the production of cAMP as the only intracellular signaling event triggered reproducibly by osteocalcin in these two cell types (Figure 2). We interpreted this result as suggesting that the, or an, osteocalcin receptor is probably a G protein coupled receptor (GPCR) linked to adenylate cyclase. Therefore, in the second step of this experimental strategy, taking advantage of the dichotomy of the functions of osteocalcin between males and females, we asked whether there were orphan GPCRs expressed at a higher level (5-fold higher) in testes than in ovaries. Out of more than a hundred orphan GPCRs submitted to this test, twenty two of them were more expressed in testes than in ovaries and only four were expressed predominantly or only in Leydig cells. One of these four orphan GPCRs, Gprc6a, was a particularly good candidate to be an osteocalcin receptor, since its inactivation in mice results in metabolic and reproductive phenotypes similar to those seen in Osteocalcin−/− mice. Furthermore, and although it was never tested through any binding assays, it has been proposed that Gprc6a was a calcium sensing receptor working better in the presence of osteocalcin.

Although the aforementioned result could not be reproduced, several criteria formally identified Gprc6a as an osteocalcin receptor present in Leydig cells. First, there is direct binding of osteocalcin to WT but not to Gprc6a-deficient Leydig cells; second, osteocalcin increases cAMP production in WT but not in Gprc6a-deficient Leydig cells; third, and more poignantly, a Leydig cell-specific deletion of Gprc6a revealed a reproduction phenotype caused by low testosterone production that was similar if not identical to the one seen in the case of osteocalcin inactivation; fourth, in an even more convincing experiment compound heterozygous mice lacking one copy of Osteocalcin and one copy of Gprc6a had a reproduction phenotype identical in all aspects to the one seen in Osteocalcin−/− or Gprc6a−/− mice. The identification of Gprc6a led subsequently to the realization that CREB is a transcriptional effector of osteocalcin regulation of testosterone biosynthesis by favoring the expression of key enzymes of this biosynthetic pathway in Leydig cells (Figure 2). The identification of Gprc6a now allows addressing many more questions; chief among them is that we may be in a position to identify functions of osteocalcin. It allows also to perform a more sophisticated dissection of osteocalcin’s molecular mode of action in known and yet to be identified target cells.

**Osteocalcin regulates male fertility independently of the hypothalamo-pituitary-axis**

The main endocrine pathway regulating male fertility is the hypothalamo-pituitary axis, in which luteinizing hormone (LH), a heterodimer between an α-subunit common to several peptide hormones and a β-subunit specific to LH favors testosterone biosynthesis. Although less severe, the reproductive phenotype of Osteocalcin−/− and Gprc6a−/− male mice bears resemblance to the one seen in Lhb−/− (LH-deficient) male mice as they are both characterized by a defect in testosterone synthesis and testosterone-dependent events. Yet, a remarkable feature of the reproduction phenotype observed in Osteocalcin−/− or Gprc6a−/− mice is that it develops in the face of an increase in circulating levels of LH. This situation raised the following question: Does osteocalcin act downstream of LH or does the realization that osteocalcin regulates male fertility reveal the existence of two different pathways, both necessary for male fertility, one pituitary-dependent and one bone-dependent?
Addressing this question, it was shown first that circulating levels of the active form (undercarboxylated) of osteocalcin were not lower in Lhb−/− than in WT male mice and daily injections of osteocalcin for one month in 6 week-old Lhb−/− male mice did not normalize circulating testosterone levels \(^{49}\). Second, histological analysis of testes of 10 week-old Lhb−/− male mice injected with osteocalcin failed to show any improvement in spermatogenesis, testis size and weight or a reversal of their Leydig cells hypoplasia \(^{49}\). Taken together these experiments indicate that the regulation of testosterone synthesis by osteocalcin does not depend on a measurable influence of Gprc6a on \(Lh\) expression.

Conceivably however, LH could be required for osteocalcin stimulation of testosterone biosynthesis by Leydig cells. Yet, two experimental evidences suggested that this is not the case. First, the positive effect of osteocalcin on testosterone synthesis in Leydig cells was recorded when cells were maintained in serum free medium, i.e., in total absence of LH \(^{49}\). Second, in cell culture LH does not regulate expression of Osteocalcin or the gene modifying it in osteoblasts. In summary these results support the notion that osteocalcin regulates male fertility independently of the hypothalamo-pituitary-axis, they also failed to provide any evidence that LH regulates Osteocalcin expression \(^{49}\).

**Bone resorption as a determinant of osteocalcin reproductive function in the mouse**

Dissociating pituitary-dependent from bone-dependent regulation of male fertility suggests the existence of a second axis regulating this function and raises the question of the identity of upstream regulators of osteocalcin reproductive function. That the ability of osteocalcin to favor glucose homeostasis is determined by osteoclastic bone resorption raised the question of whether male fertility was another physiological function to be added to the credit of bone resorption \(^{23}\). Testing this hypothesis using loss-of-function and gain-of-function mouse models of bone resorption, it was shown that bone resorption is a physiological determinant of osteocalcin’s regulation of testosterone production and male reproductive function through its ability to activate osteocalcin \(^{49}\). In addition, it was recently shown in humans that osteocalcin and the bone turnover is associated with testosterone circulating levels in the general population and in patients with bone disorders \(^{40,41}\). The data presented so far support a model in which osteoclast-mediated bone resorption regulates male fertility in mice through the decarboxylation and the activation of osteocalcin. This model combined with previously published observations also implies that bone resorption is required not only for male fertility, but also for the control of energy metabolism \(^{22-24}\).

The cardinal role of bone resorption in the regulation of testosterone production provided the opportunity to look for these additional upstream regulators of osteocalcin reproductive function. As described previously, insulin signaling in osteoblasts enhances osteocalcin activity, which in turn favors insulin secretion. Consequently, insulin signaling in osteoblasts might influence testosterone biosynthesis in an osteocalcin-dependent manner \(^{49}\).

This possibility was addressed by analyzing male mice lacking the gene encoding the insulin receptor selectively in osteoblasts (InsR\(_{osb}^{-/-}\)) \(^{23}\). These animals, that have less active osteocalcin, demonstrated a decrease in testes size and weight, both in epididymides and seminal vesicle weights and in sperm count and circulating testosterone levels. Lastly, this observation was firmly confirmed by generating and testing compound mutant mice lacking one allele of InsR in osteoblasts and one allele of either Osteocalcin or Gprc6a. Here again, testis, epididymides, and seminal vesicle weights, and sperm count and circulating levels of testosterone, in these compound mutant mice demonstrated abnormalities that were similar to those seen in InsR\(_{osb}^{-/-}\), Osteocalcin−/− or even greater relevance, in Gprc6a−/− mice \(^{30,32,49}\). Taken together, these observations strongly suggested the existence of a
pancreas-bone-testis axis in the control of male reproductive functions that acts in parallel to the hypothalamus-pituitary-testis axis \(^{(49)}\) (Figure 3).

**Conservation of the reproductive function of osteocalcin in human**

A second legitimate question that has plagued osteocalcin research since its recognition as a hormone in rodents has been to determine whether it also has an endocrine function in humans. The function of osteocalcin as a regulator of testosterone production has been recently extended to humans. It was shown that osteocalcin and the bone turnover is associated with testosterone circulating levels in general population and in patients with bone disorders \(^{(40)}\). Moreover, Dr. Khosla’s group has also shown that there is a significant association between serum osteocalcin and testosterone levels during mid-puberty in males \(^{(41)}\). They postulate that this axis may be most relevant during rapid skeletal growth in adolescent human males to help maximize bone size. However, while there is a growing body of evidence that osteocalcin serum levels are a reliable indicator of the degree of insulin secretion, insulin sensitivity and circulating serum testosterone levels in humans \(^{(42-44)}\), until recently, there was no genetic evidence establishing that osteocalcin fulfills its endocrine functions in humans.

The identification of an osteocalcin receptor and the realization that osteocalcin influences male fertility, provided an opportunity to tackle this issue. In fact, the fertility phenotype of the \(Osteocalcin^{-/-}\) mice, mainly characterized by a subfertility, mediocre spermogram, low circulating testosterone levels and high circulating LH levels \(^{(30)}\), is the exact phenocopy of a rare but well defined syndrome in human called peripheral testicular insufficiency \(^{(45-48)}\). Thus, a systematic genomic analysis of \(Osteocalcin\) and \(GPRC6A\) loci of a cohort of 59 patients with this syndrome was initiated with the goal to identify loss-of-function mutation in \(Osteocalcin\) or its receptor that would explain their clinical presentation \(^{(49)}\).

As a result of this genomic analysis, two patients in this cohort harbored a point mutation T>A transversion in exon 4 of GPRC6A \(^{(49)}\). This missense mutation results in an amino acid substitution (F464Y) in a highly conserved region of one of the transmembrane domain of GPRC6A and prevents its localization to the cell membrane, therefore resulting in a loss of function of this receptor \(^{(49)}\). Furthermore, three different cell-based assays indicated that the mutation also acts in a dominant negative manner in cells \(^{(49)}\). Lastly, it was noted that both patients harboring this substitution-mutation in \(GPRC6A\) originated from the same region of the globe, shared a history of glucose intolerance and displayed similar defects in reproductive hormones. Indeed, these patients presented a metabolic syndrome characterized by an increase in body mass index, as well as a glucose intolerance determined by hyperinsulinemia after fasting, a glucose tolerance test, and an insulin tolerance test \(^{(49)}\). Many of these features are seen in mice lacking \(Osteocalcin\) or \(Gprc6a\) in all cells \(^{(7,30,32-34,47)}\). It is thus interesting that the F464Y variant was not observed in 1,000 controls. Careful phenotypic analysis of individuals carrying the F464Y allele may clarify the spectrum of associated metabolic, cardiovascular and reproductive defects. Taken together these results indicate the importance of osteocalcin signaling in human and suggest that GPRC6A may be a new susceptibility locus for primary testicular failure in humans, a disease whose cause is often un-indentified. Results of this initial foray in the genetic analysis of osteocalcin functions in humans should be viewed as a stepping-stone to perform a more systematic analysis in a larger patient population with primary testicular failure as well as in patients with glucose intolerance or metabolic syndromes.

The known functions of the skeleton indicate that skeleton physiology affects many more organs and functions than just skeleton itself, as it affects glucose homeostasis, energy expenditure and fertility. These novel functions of bone underscore the notion that the
skeleton is an important member of the endocrine network affecting multiple functions in the body. Moreover, they also raise the strong possibility that other endocrine functions of the skeleton have yet to be described.

References


Highlights: “Regulation of male fertility by the bone-derived hormone osteocalcin”

- A novel dimension to the bone physiology: the skeleton secretes two hormones
- Osteocalcin, a bone-derived hormone, favors testosterone production by the Leydig cells of the testis
- Osteocalcin regulates male fertility independently of the hypothalamo-pituitary-axis
- Bone resorption as a determinant of osteocalcin reproductive function in the mouse
- Conservation of the reproductive function of osteocalcin in human
Figure 1. The skeleton is an endocrine organ
The endocrine regulation of energy metabolism and male reproduction by the bone is mediated by osteocalcin, an osteoblast-specific secreted molecule. Osteocalcin regulates energy metabolism by increasing insulin secretion, favoring pancreatic-β-cell proliferation, and increasing insulin sensitivity in various tissues. In addition, it promotes male reproductive function by stimulating testosterone synthesis in Leydig cells.
Osteocalcin has an unusual mode of activation that relies on the interplay between two specific bone cells: Osteoblasts and Osteoclasts. The osteoblasts produce and secrete an inactive form of this molecule (carboxylated) that is stored in the extracellular bone matrix (ECM). The activity of the osteoclasts create resorption lacunae in the ECM inducing a low PH (4.5) which is necessary and sufficient to bio-activate osteocalcin by promoting its undercarboxylation. The mechanism by which osteocalcin is activated is regulated in osteoblasts by insulin signaling, which inhibits the expression in osteoblasts of the gene encoding osteoprotegerin (Opg), which hampers osteoclast differentiation. Following its binding to Gprc6a expressed in Leydig cells, osteocalcin favors cAMP production that leads to the activation of the transcription factor CREB (cAMP response element binding). CREB activates the expression of several genes encoding the enzymes that are necessary for testosterone biosynthesis, such as StAR, Cyp11a, 3-HSD and Cyp17. Steroidogenic acute regulatory protein (StAR) is crucial for transport of cholesterol to mitochondria where biosynthesis of steroids is initiated. Cyp11a encodes the cholesterol side-chain cleavage enzyme (P450scc) that catalyzes the first and rate-limiting step, which converts cholesterol to pregnenolone. 3-HSD and Cyp17 encode two enzymes required during the conversion of pregnenolone to testosterone. Testosterone is a sex steroid hormone require for many aspects of testicular functions, such as germ cell survival and spermatogenesis.
Figure 3. Osteocalcin-stimulated testosterone biosynthesis is positively regulated by insulin signaling in osteoblasts

Insulin signaling in osteoblasts stimulates the bio-activation of osteocalcin. In a feedback loop control, undercarboxylated active osteocalcin then stimulates insulin secretion by the cells of the pancreatic islets, promotes insulin sensitivity in peripheral organs and favors testosterone biosynthesis in Leydig cells of the testis. Testosterone in turn favors bone growth, maintenance and maturation.