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## Endocrine regulation of male fertility by the skeleton

Franck Oury<sup>1,\*</sup>, Grzegorz Sumara<sup>1,\*</sup>, Olga Sumara<sup>1</sup>, Mathieu Ferron<sup>1</sup>, Haixin Chang<sup>2</sup>, Charles E. Smith<sup>3</sup>, Louis Hermo<sup>3</sup>, Susan Suarez<sup>2</sup>, Bryan L. Roth<sup>4</sup>, Patricia Ducy<sup>5</sup>, and Gerard Karsenty<sup>1,‡</sup>

<sup>1</sup>Department of Genetics & Development, Columbia University, New York, NY 10032, USA

<sup>2</sup>Department of Biomedical Sciences, Cornell University, T5-006 Veterinary Research Tower, Ithaca, NY 14853, USA

<sup>3</sup>Department of Anatomy and Cell Biology, McGill University, 3640 University St, Montreal, Quebec, H3A 2B2, Canada

<sup>4</sup>Department of Pharmacology, Medicinal Chemistry and Natural Products and National Institute of Mental Health Psychoactive Drug Screening Program, University of North Carolina, Schools of Medicine and Pharmacy, Chapel Hill, NC 27599, USA

<sup>5</sup>Department of Pathology & Cell Biology, Columbia University, New York, NY 10032, USA

### Abstract

Although the endocrine capacity of bone is widely recognized, interactions between bone and the reproductive system have until now focused on the gonads as a regulator of bone remodeling. We now show that in males, bone acts as a regulator of fertility. Using co-culture assays, we demonstrate that osteoblasts are able to induce testosterone production by the testes, while they fail to influence estrogen production by the ovaries. Analyses of cell-specific loss- and gain-of-function models reveal that the osteoblast-derived hormone osteocalcin performs this endocrine function. By binding to a G-protein coupled receptor expressed in the Leydig cells of the testes, osteocalcin regulates in a CREB-dependent manner the expression of enzymes required for testosterone synthesis, promoting germ cell survival. This study expands the physiological repertoire of osteocalcin, and provides the first evidence that the skeleton is an endocrine regulator of reproduction.

### Introduction

Bone is a dynamic tissue undergoing modeling during childhood and remodeling throughout adulthood (Harada and Rodan, 2003; Rodan and Martin, 2000). These two processes, referred thereafter as bone (re)modeling, are characterized by the succession of resorption of mineralized bone by osteoclasts and *de novo* formation by osteoblasts. Bone (re)modeling is regulated locally by cytokines produced by bone cells and systemically by hormones and neuropeptides (Harada and Rodan, 2003; Karsenty et al., 2009). One of the most powerful hormonal regulations of bone (re)modeling is exerted by the sex steroid hormones that are

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‡Corresponding Author: gk2172@columbia.edu.

\*These two authors equally contributed to this work

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necessary to maintain bone integrity (Khosla et al., 2001; Nakamura et al., 2007; Riggs et al., 1998). The biological importance of this regulation is best exemplified by the fact that gonadal failure triggers bone loss and causes osteoporosis in post-menopausal women (Manolagas et al., 2002; Rodan and Martin, 2000). To date the study of the interplay between gonads and bone has focused on the mechanism whereby sex steroid hormones affect bone mass accrual (Manolagas et al., 2002; Nakamura et al., 2007).

Based on physiological and clinical observations, we hypothesized ten years ago that bone mass, energy metabolism and reproduction might be coordinately regulated (Ducy et al., 2000). Testing this hypothesis revealed that bone is an endocrine organ favoring whole-body glucose homeostasis and energy expenditure. These novel functions of bone are mediated by an osteoblast-specific secreted molecule, osteocalcin, that when uncarboxylated acts as a hormone favoring  $\beta$  cell proliferation, insulin secretion and sensitivity and energy expenditure (Lee et al., 2007). A second gene expressed in osteoblasts, *Esp*, inhibits endocrine functions of osteocalcin by favoring, through an indirect mechanism, its carboxylation (Ferron et al., 2010; Fulzele et al., 2010). Despite these findings, basic facts about how osteocalcin performs its endocrine function are unknown. Most importantly, the receptor for osteocalcin remains to be determined, as do the signaling pathways triggered by this hormone in target cells.

We now show that osteocalcin, in addition to its endocrine role as a regulator of energy homeostasis, favors male fertility. It does so by promoting synthesis by Leydig cells of testosterone, a steroid hormone required for many aspects of testicular function (Sinha Hikim and Swerdloff, 1999; Walker, 2009), and has no effect on female fertility. Furthermore, we identify a bona fide receptor for osteocalcin that is expressed and transduces its signal in Leydig cells. Using this tool we identify genes whose expression is regulated by osteocalcin in these cells and that account for its regulation of male fertility. Our findings expand the biological importance of osteocalcin, begin to unravel its molecular mode of action, and provide the first evidence that the skeleton is an endocrine regulator of fertility.

## Results

### Osteoblasts enhance testosterone production by Leydig cells

In an effort to determine whether osteoblasts or any other cells of mesenchymal origin may regulate the functions of gonads we asked whether supernatants of mesenchymal cell cultures affect hormone production by testes and/or ovaries (Figure 1A).

In a first set of exploratory experiments we observed that of all those tested, the supernatant of osteoblast cultures increased testosterone secretion by testes explants to the largest extent (over 3 fold), while not affecting estradiol and progesterone secretion by testes or ovaries (Figure 1B–G). In subsequent experiments testes explants were replaced by defined cell populations. Since testosterone is produced by Leydig cells we asked whether osteoblast-derived molecule(s) act(s) directly on Leydig cells by culturing mouse primary Leydig cells in the presence or absence of supernatants of osteoblast cultures or of other mesenchymal cell types cultures. In the conditions of this assay, supernatants of osteoblast cultures were also the only ones able to significantly increase testosterone production by Leydig cells (more than 4 fold) (Figure 1H). These experiments indicate that osteoblasts are the cells of mesenchymal origin that affect testosterone biosynthesis to the largest extent, and that they do so through secreted molecule(s) acting on Leydig cells of the testis. This novel endocrine function of osteoblasts was restricted to androgen production.

## Osteocalcin favors male fertility by enhancing testosterone production

Osteocalcin is a major osteoblast-derived hormone. We had previously noticed that *Osteocalcin*-deficient male mice (*Ocn*<sup>-/-</sup> breed poorly (P.D. and G.K. unpublished observation), and so we tested whether it could be a, or the, osteoblast-derived hormone enhancing testosterone secretion by Leydig cells.

Several lines of evidence indicated that it is the case. First, supernatants of wild type (WT) but not of *Ocn*<sup>-/-</sup> osteoblast cultures increased testosterone production by Leydig cells (Figure 2A). Second, treating Leydig cells with increasing amount of uncarboxylated osteocalcin, the active form of the hormone, resulted in a dose-dependent increase in testosterone secretion, although at high concentration the stimulatory effect of osteocalcin weakened (Figure 2B). Third, injection of osteocalcin in WT mice increased circulating levels of testosterone (Figure 2C). Fourth, we analyzed loss- (*Ocn*<sup>-/-</sup> mice) and gain-of-function (*Esp*<sup>-/-</sup> mice) mouse models for osteocalcin (Lee et al., 2007). When *Ocn*<sup>-/-</sup> males were crossed with WT female mice, the size of the litters was nearly twofold smaller than when WT males were crossed with WT females (Figure 2D). Conversely, the number of pups per litter was increased when *Esp*<sup>-/-</sup> males were bred with WT female mice, although this increase did not reach statistical significance (Figure 2D). The frequency of litters over a period of 8 weeks was also decreased in the case of the loss-of-function model and increased in the gain-of-function model (Figure 2E). Testis size and weight were significantly decreased in *Ocn*<sup>-/-</sup> and increased in *Esp*<sup>-/-</sup> mice at 3 months of age. In some of the latter mutant mice this was caused, in part, by fluid accumulation (Figure 2F–G). The weights of epididymides and seminal vesicles and sperm count were also significantly decreased in *Ocn*<sup>-/-</sup> and increased in *Esp*<sup>-/-</sup> mice (Figure 2H–J). These abnormalities worsened over time (Figures 2G and 2J).

Motility of sperm from both WT and *Ocn*<sup>-/-</sup> males was assessed by videomicroscopy immediately after dissemination from the caudal epididymis or after 2 hours of incubation under conditions known to prepare sperm for fertilization (Suarez and Osman, 1987). In both cases, the percentage of motile sperm did not differ between *Ocn*<sup>-/-</sup> and WT mice (Figure S1A). Likewise, the percentage of abnormally shaped or dead sperm was similar in WT and *Ocn*<sup>-/-</sup> mice (Figure S1B–C).

Consistent with the fact that osteocalcin favors testosterone synthesis in Leydig cells *ex vivo*, circulating levels of testosterone were markedly decreased in *Ocn*<sup>-/-</sup> and increased in *Esp*<sup>-/-</sup> mice at all time points tested (Figure 2K). Accordingly, circulating levels of luteinizing hormone (LH), a pituitary-derived hormone favoring testosterone synthesis, was increased 2.5 fold in *Ocn*<sup>-/-</sup> mice (Figure 2L). Taken together these cell biological and genetic experiments identify osteocalcin as a secreted molecule favoring male fertility by increasing testosterone production by Leydig cells.

Circulating progesterone levels were similar in *Ocn*<sup>-/-</sup> and WT mice and, although circulating levels of estradiol were higher in *Ocn*<sup>-/-</sup> than in WT mice, they remained within the normal range (from 9.3 to 28.9 ng/ml for non-breeder mice and from 14.4 to 71.1 ng/ml for breeder mice) (Figure 2K). Estradiol levels were not affected in *Esp*<sup>-/-</sup> mice. As predicted by the co-culture assays, female fertility, ovary weight, morphology of the uterus, follicles number and circulating levels of sex steroid hormones were normal in *Ocn*<sup>-/-</sup> female mice (Figure S1D–L).

## Osteocalcin regulates male fertility as an osteoblast-derived hormone

To verify that osteocalcin regulates male fertility as an osteoblast-secreted hormone and not as a testis-secreted factor, we performed gene expression and cell-specific gene deletion experiments.

When assessing *Osteocalcin* expression by quantitative PCR (qPCR) we observed that it was more than 750 fold higher in bone than in gonads; accordingly, we failed to detect *Osteocalcin* transcript or protein in testes by *in situ* hybridization or Western blot analyses (Figure 3A–C). To be able to trace *Osteocalcin*-expressing cells *in vivo* we knocked the *mCherry* fluorescent reporter gene into the *Ocn* locus (*Ocn-mCherry* mice) (Figure S2A–B). While we observed the expected strong signal in osteoblasts, there was no detectable *mCherry* fluorescence in testes (Figure 3D). Thus in multiple assays, we failed to detect *Osteocalcin* expression in testes.

Next, we generated cell-specific loss- and gain-of-function models of osteocalcin by crossing mice harboring floxed alleles of *Ocn* (Figure S2C–D) or *Esp* with either the *al1* *Collagen-Cre* transgenic mice or the *Cyp17-iCre* transgenic mice to delete genes in osteoblasts or in Leydig cells only, respectively (Bridges et al., 2008; Dacquin et al., 2002). Testes size and weight, epididymides and seminal vesicles weights, sperm count and circulating testosterone levels were all reduced in 12 week-old *Ocn<sub>osb</sub><sup>-/-</sup>* mice. None of these parameters were affected in mice lacking *Osteocalcin* in Leydig cells only (Figure 3E–I). There was a tight correlation between circulating levels of osteocalcin and testosterone in *Ocn<sub>osb</sub><sup>-/-</sup>* mice (Figure 3J). Conversely, *Esp<sub>osb</sub><sup>-/-</sup>* mice displayed testicular abnormalities identical to those of *Esp<sup>-/-</sup>* mice. Inactivation of *Esp* in Sertoli cells, where this gene is expressed (Dacquin et al., 2004; Jamin et al., 2003), had no detectable deleterious consequence on testis biology demonstrating that it is through its expression in osteoblasts, not in Sertoli cells, that *Esp* regulates male fertility (Figure 3K–N). These experiments therefore indicate that it is only through its expression in osteoblasts that osteocalcin promotes male fertility.

### Cellular and molecular bases of osteocalcin regulation of male fertility

To address this aspect of osteocalcin biology, we first studied the morphology of Leydig cells by immunostaining of 3-beta-hydroxysteroid dehydrogenase (3β-HSD). The number of Leydig cells was not significantly affected by the absence of osteocalcin or *Esp*, nor was the expression of genes affecting cell proliferation (Figure 4A and data not shown). However, Leydig cells appeared hypotrophic in *Ocn<sup>-/-</sup>* testes as determined by the significant decrease of the ratio between the Leydig cells and interstitial areas observed in *Ocn<sup>-/-</sup>* compared to WT testes (Figure 4B–C). Conversely, this ratio was increased in *Esp<sup>-/-</sup>* testes (Figure 4B–C).

When germ cells were analyzed through a stereological approach, we observed that the number of spermatocytes and round spermatids was significantly decreased in *Ocn<sup>-/-</sup>* mice (Figure 4D). Consistently, the size of the epithelium in testis tubules was also significantly decreased in these mutant mice, a feature suggesting that osteocalcin regulates, presumably through its effect on testosterone biosynthesis, germ cell number (Figure S3B). Since testosterone inhibits germ cell apoptosis (Brinkworth et al., 1995; Henriksen et al., 1995; Sinha Hikim and Swerdloff, 1999) we performed TUNEL assays. Those showed a 2 fold increase in germ cell apoptosis in *Ocn<sup>-/-</sup>* compared to WT mice and a 2 fold decrease in *Esp<sup>-/-</sup>* testes (Figure 4F). Importantly, there was an increase in the number of apoptotic cells in stages VI–VIII of the spermatogenic cycle, the very stages of spermatogenesis in which testosterone affects germ cell apoptosis more efficiently (Sharpe et al., 1992) (Figure S3C). There were on the other hand no abnormalities of germ cell proliferation in either *Ocn<sup>-/-</sup>* or *Esp<sup>-/-</sup>* mice (Figure S3A). Taken together these data further indicate that the decrease in fertility demonstrated by *Ocn<sup>-/-</sup>* male mice is caused by a decrease in testosterone levels.

To determine how osteocalcin favors testosterone synthesis by Leydig cells we tested whether it affects expression of the enzymes necessary for testosterone biosynthesis such as

*StAR*, *Cyp11a*, *Cyp17* and *3 $\beta$ -HSD*. In cell culture, uncarboxylated osteocalcin increased expression of these genes in Leydig cells (Figure 4F). Accordingly, their expression was significantly decreased in *Ocn*<sup>-/-</sup> and increased in *Esp*<sup>-/-</sup> testes (Figure 4G–H), while it was unaffected in *Ocn*<sup>-/-</sup> and *Esp*<sup>-/-</sup> ovaries or adrenal glands (Figure S3D–G). Of note, there was no change in expression of *Cyp19*, the gene encoding the testosterone aromatase, or of *HSD-17* in *Ocn*<sup>-/-</sup> and *Esp*<sup>-/-</sup> testes (Figure 4F–H).

To further support the notion that osteocalcin influences germ cell apoptosis through testosterone, we examined expression of *Gonadotropin Regulated Testicular Helicase* (*Grth*) because this gene has emerged as an essential regulator of spermatogenesis that inhibits germ cell apoptosis and whose expression in germ cells and Leydig cells is regulated by testosterone (Dufau and Tsai-Morris, 2007; Tsai-Morris et al., 2007; Tsai-Morris et al., 2010). *Grth* expression was decreased in *Ocn*<sup>-/-</sup> and increased in *Esp*<sup>-/-</sup> testes (Figure 4I). GRTH inhibits activation of Caspase 3, a determinant of apoptosis (Sheng et al., 2006), and favors expression of tACE, a protein favoring germ cell maturation. Consistent with these notions, Western blot analyses showed an increase of cleaved caspase 3 accumulation and a decrease of tACE in *Ocn*<sup>-/-</sup> testes (Figure 4J).

### **Gprc6a, a G-protein coupled receptor, transduces osteocalcin signal in Leydig cells**

To begin to elucidate the molecular mode of action of osteocalcin, we next searched for a receptor expressed in Leydig cells that could transduce its signal. To that end we used a two-step experimental strategy taking advantage of the fact that osteocalcin regulates fertility only in male mice.

First, we defined the signal transduction pathway used by osteocalcin (these experiments were performed in TM3 cells and not primary Leydig cells, in order to obtain a sufficient amount of extract for analysis). With this aim, we treated the cells with uncarboxylated osteocalcin and assayed for tyrosine phosphorylation, ERK activation, intracellular calcium accumulation and cAMP production using in each case an appropriate positive control. Osteocalcin consistently induced cAMP production in Leydig cells to a level comparable to that induced by human chorionic gonadotropin, the positive control, but did not induce tyrosine phosphorylation, ERK activation or intracellular calcium accumulation in these cells (Figure 5A–D). At higher concentrations, osteocalcin stimulation of cAMP production weakened. These data implied that the osteocalcin receptor may be a G-protein coupled receptor (GPCR). Hence, in the second step of this experimental strategy we took advantage of the dichotomy of function of osteocalcin between males and females and asked how many orphan GPCRs were expressed in testis at a level at least 5 fold higher than in ovary. Twenty-two out of 103 orphan GPCRs tested were predominantly expressed in testes; out of these 22, 4 were enriched in Leydig cells (Figure 5E–F). Among them, *Gprc6a* caught our attention because its deletion in all cells results in a metabolic and fertility phenotype similar to that of *Ocn*<sup>-/-</sup> mice (Pi et al., 2008).

Immunofluorescence experiments verified that *Gprc6a* is expressed in Leydig cells in testes but not in follicular cells of the ovary. Importantly, the same is true in human gonads (Figure 5G–H and Figure S4B). Post-natally, *Gprc6a* expression peaked within the first week of life, when circulating testosterone levels are elevated. *Gprc6a* expression then decreased, before increasing again at 6 weeks of age when circulating levels of testosterone also rebound (Figure 5I). We also performed binding assays on mouse testes using biotinylated osteocalcin as a ligand. In the conditions of this assay osteocalcin bound to Leydig cells and the specificity of this binding was confirmed by several criteria. First, there was no signal when using avidin-biotin alone; second, there was no signal either in other cellular compartments of the testicular tubules; third, we could not detect any binding when using *Gprc6a*-deficient testes; fourth, osteocalcin binding could be competed away by an excess



(100 fold) of unlabeled osteocalcin but not by the same excess of hCG or of other molecules proposed as ligands of Gprc6a (Wellendorph et al., 2005) (Figure 5J). These data identify Gprc6a as an osteocalcin receptor in Leydig cells.

To define Gprc6a function in Leydig cells *in vivo*, we generated *Gprc6a<sup>Leydig</sup><sup>-/-</sup>* mice. Prior to analyzing these *Gprc6a<sup>Leydig</sup><sup>-/-</sup>* mice we verified that we had deleted *Gprc6a*, albeit partially (75% of deletion), in Leydig cells but not in other organs (Figure S5C–D). In *Gprc6a<sup>Leydig</sup><sup>-/-</sup>* male mice, testes size and weight, epididymis and seminal vesicle weights, sperm counts and circulating testosterone levels, and Leydig cell area were all reduced, as was the expression of *Grth* and the 3 genes controlling testosterone biosynthesis that are regulated by osteocalcin (Figure 6A–I and Figure S5E). Accordingly, the number of apoptotic germ cells was increased compared to WT testes (Figure 6J). To establish genetically that Gprc6a is a signaling receptor for osteocalcin in Leydig cells, we analyzed compound mutant mice lacking one allele of *Ocn* and one allele of *Gprc6a* in Leydig cells only (*Ocn<sup>+/-</sup>;Gprc6a<sup>Leydig</sup><sup>+/-</sup>* mice). Whether we looked at testes, epididymis and seminal vesicle weights or sperm count *Ocn<sup>+/-</sup>;Gprc6a<sup>Leydig</sup><sup>+/-</sup>* mice had a phenotype identical to that observed in *Gprc6a<sup>Leydig</sup><sup>-/-</sup>* and *Ocn<sup>osb</sup><sup>-/-</sup>* mice (Figure 6A–I).

### CREB is a transcriptional effector of osteocalcin signaling in Leydig cells

Observations that osteocalcin treatment of Leydig cells increased cAMP production and that the osteocalcin receptor is a GPCR suggested that CREB could mediate osteocalcin functions in these cells. In support of this hypothesis, osteocalcin treatment of Leydig cells favors CREB phosphorylation (Figure 7A). To investigate this further, mice were generated that lack CREB expression specifically in Leydig cells (*Creb<sup>Leydig</sup><sup>-/-</sup>* mice).

Twelve week-old *Creb<sup>Leydig</sup><sup>-/-</sup>* male mice displayed a reduction in testis size and weight, in epididymides and seminal vesicles weights, in sperm count and in circulating testosterone levels similar to those seen in *Ocn<sup>-/-</sup>* and *Gprc6a<sup>Leydig</sup><sup>-/-</sup>* mice (Figure 7B–G and Figure S5A). *Creb<sup>Leydig</sup><sup>-/-</sup>* mice also demonstrated a strong decrease in the expression of *Grth* and of the 4 genes involved in testosterone biosynthesis whose expression is regulated by osteocalcin (Figure 7H–I). In agreement with these data, CREB could bind to the promoter regions of *Cyp11a*, *3β-HSD* and *StAR* (Zhang et al., 2005) (Figure 7J). To establish whether CREB acts downstream of Gprc6a in Leydig cells to regulate male fertility, we generated compound heterozygous mice lacking one copy of *Creb* and one copy of *Gprc6a* in Leydig cells. The decrease in fertility demonstrated by *Creb<sup>Leydig</sup><sup>+/-</sup>;Gprc6a<sup>Leydig</sup><sup>+/-</sup>* male mice was similar to that observed in *Creb<sup>Leydig</sup><sup>-/-</sup>* or *Gprc6a<sup>Leydig</sup><sup>-/-</sup>* male mice (Figure 7B–F and 7H) and was not observed in single heterozygous mutant mice (data not shown). These results identify CREB as a transcriptional mediator of osteocalcin regulation of testosterone biosynthesis in Leydig cells.

## Discussion

This study reveals that bone is a positive regulator of male fertility. This interaction is mediated through the osteoblast-derived hormone osteocalcin, which binds to a specific receptor present on Leydig cells of the testis and favors testosterone biosynthesis (Figure 7K). Our results, along with those previously published support the hypothesis that regulation of bone remodeling, energy metabolism and reproduction are linked (Ducy et al., 2000; Lee et al., 2007). They also demonstrate that bone is a more important regulator of whole-organism physiology than was anticipated.

In the last ten years, the view of bone as a mere assembly of inert calcified tubes has evolved because two independent lines of investigation painted a much more dynamic picture of this tissue. Firstly, the dialogue between bone physiology and energy metabolism and the

paramount influence of the brain in the control of bone mass accrual became apparent (Karsenty, 2006). Secondly, it has been realized that bone is an endocrine organ regulating at least two functions, phosphate metabolism and energy homeostasis, through two distinct osteoblast-derived hormones, FGF23 and osteocalcin (Fukumoto and Yamashita, 2007; Lee et al., 2007). These two endocrine functions of bone begged the following question: are these the only functions affected by bone in its endocrine capacity?

The well-known regulation of bone remodeling by gonads provides an ideal setting to address this question, and raises the possibility that bone, in its endocrine capacity, could influence through a feedback mechanism reproductive functions in either gender. We show here that osteoblasts, the bone forming cells, favor through osteocalcin fertility in male but not in female mice. Our observation that *Ocn*<sup>-/-</sup> mice have low circulating testosterone levels despite an increase in circulating LH levels may have several explanations. For instance, it could be that deletion of osteocalcin causes, for unknown reasons, a loss of negative feedback. Alternatively it may also suggest that LH cannot favor testosterone production in the absence of osteocalcin. Further experiments will be required to address this point.

All of our experiments, conducted *ex vivo* and *in vivo* in loss- and gain-of-function models, indicate that the main mechanism whereby osteocalcin favors male fertility is by increasing testosterone synthesis in Leydig cells. Testosterone in turn supports maturation and prevents apoptosis of germ cells (Henriksen et al., 1995; Sinha Hikim and Swerdloff, 1999; Walker, 2009). Since there is no expression of *Gprc6a* in Sertoli cells or germ cells, it is likely that osteocalcin regulates male fertility by binding to its receptor on Leydig cells. This was confirmed by the analysis of the Leydig cell-specific deletion of *Gprc6a* and *Creb*. Testosterone affects germ cell survival by involving other cell types such as Sertoli cells, since germ cells do not express androgen receptor (Bremner et al., 1994; De Gendt et al., 2004; Wang et al., 2003). Unexpectedly, *Ocn*<sup>-/-</sup> male mice have a higher level of circulating estrogen than WT littermates, even though osteocalcin does not promote estradiol synthesis. The most likely explanation for this mild increase in circulating estradiol levels in the *Ocn*<sup>-/-</sup> mice is that the increase in the number of adipocytes caused by *Osteocalcin* inactivation may result in an increase in the aromatization of testosterone into estrogen in fat (Nelson and Bulun, 2001; Simpson et al., 2000; Simpson, 2003). Importantly, this was also observed in mice lacking *Gprc6a* (Pi et al., 2008).

The existence of such a profound influence exerted by a hormone other than LH on sex steroid hormone synthesis raises the question of whether it is a male-specific phenomenon. To date we have no evidence that, at least in the mouse, the skeleton favors estrogen production in females. This however does not rule out the possibility that other peripheral organs may secrete hormones favoring estrogen synthesis.

The growing number of functions identified for osteocalcin makes the identification of its receptor all the more important. An unbiased approach based on the ability of osteocalcin to increase cAMP production in Leydig cells and on its dichotomy of function between male and female gonads led to the identification of *Gprc6a* as the osteocalcin receptor. This orphan receptor, which belongs to the C family of GPCRs (Wellendorph and Brauner-Osborne, 2004), has been proposed to be a receptor for amino acids, for calcium in the presence of osteocalcin as a cofactor and for androgen (Pi et al., 2008; Pi et al., 2005; Pi et al.). Yet, the possibility that *Gprc6a* could be a specific receptor for osteocalcin has never been tested through biochemical or genetic means. Here we provide biochemical and genetic evidence establishing its identity as a specific receptor for osteocalcin in Leydig cells while we failed to detect an interaction between amino acids and *Gprc6a*. That *Gprc6a* is expressed in human and mice Leydig cells in the testes but not in follicular cells of ovaries

provides a molecular basis for the fact that osteocalcin affects male fertility only. The expression of this receptor is extremely dynamic and peaks at adulthood when testosterone biosynthesis is at its maximum (Feldman et al., 2002; Gray et al., 1991; Quigley, 2002). This observation suggests that the regulation of osteocalcin functions occurs, at least in part, by regulating the expression of its receptor. The identification of an osteocalcin receptor opens the door to a thorough molecular dissection of the mode of action of osteocalcin in various cell types where it is expressed. This may eventually lead to the identification of additional functions for osteocalcin.

An obvious question raised by this study is to know whether the skeleton also regulates male fertility in humans? In the absence of inactivating mutations in the *Osteocalcin* or *Gprc6a* genes in humans and of studies correlating circulating levels of uncarboxylated osteocalcin and fertility in the aging male population, this question can only be addressed through indirect means for now. Although we cannot rule out the possibility that the endocrinology of reproduction may be different in this particular aspect between rodents and humans, indirect evidence suggests that the function of osteocalcin described here may be conserved in humans. Firstly, and most importantly, this work was in part initiated because of a clinical observation made in humans: loss of sex steroid hormones triggers a decrease in bone mass. This led us to test whether feedback regulation of fertility by bone may also occur. Secondly, *Gprc6a* is expressed, in human and in mice, in Leydig cells of the testes but not in ovaries. Hence, the entire signaling cascade from the hormone to the receptor exists in the equivalent organs in humans. Third, the growing number of reports indicating that osteocalcin is a reliable indicator of glucose intolerance, just as it is in mice, strongly suggests that this molecule also acts as a hormone in humans (Kanazawa et al., 2010; Saleem et al., 2010; Yeap et al., 2010). Along these lines, there is no example yet of a molecule being a hormone in the mouse that has abruptly lost this attribute in humans. This is, nevertheless, an aspect of osteocalcin that will need further investigation in the future.

## Experimental procedures

### Mice generation

All experiments were performed on the 129-Sv (Taconic) genetic background. Control littermates were used in all experiments. Mice genotypes were determined by PCR; primer sequences are available upon request. *Osteocalcin-mCherry* knock-in, *Ocn* conditional and *Gprc6a* conditional allele generation strategies are described in Figure S2 and S5.

### Primary Leydig cells and testes explant preparation

Adult mouse Leydig cells were isolated by mechanical dissociation of the testes followed by purification on a 0–90% Percoll gradient (Hunter et al., 1982; Schumacher et al., 1978). Primary Leydig cells were cultured in Minimal Essential Medium (MEM + GlutaMAX, Invitrogen) supplemented with 1x PenStrep, 25mM HEPES, pH 7.4 and 0.07% BSA at 33°C in 5% CO<sub>2</sub>. After 3 h of attaching and starvation, cells were washed once with culture medium and then used for experiments. The testes explant preparation protocol was adapted from (Powlin et al., 1998). Explants were washed 3 times with PBS and placed in serum free RPMI medium for 2 hours before being used for experiments.

### Osteocalcin stimulation of Leydig cells or testes explants

Primary Leydig cells and testes explants were washed 3 times with PBS and stimulated with different doses of recombinant osteocalcin prepared as previously described (Ferron et al., 2008) or with hCG as a positive control. After 1 h, an aliquot of medium was collected for measurements of testosterone. Cells were then maintained for 3 additional hours and lysed in 1 ml TRIZOL (Invitrogen) for RNA isolation.



### Sperm counts and hormone measurements

Caudal epididymides were minced in 1 ml PBS, and the number of cells released was counted after 1 h. The total sperm count was assessed in the final suspension by using a hemocytometer (Dakhova et al., 2009). Circulating levels of testosterone, estradiol (E2) and progesterone were measured by radioimmunoassay (RIA) from Diagnostic Systems Laboratories (Testosterone RIA DSL-4000, Estradiol RIA DSL-43100 and Progesterone RIA-3900).

### Histology

One testis or ovary from each mouse was randomly selected for molecular analysis and the other one was used for histology. Specimens were collected, weighed and fixed in Bouin's fixative for histological analyses before being dehydrated through graded ethanol, processed for paraffin embedding and serially sectioned at 5  $\mu$ m. For histological analysis, testes and ovaries sections were stained with periodic acid-Schiff and counterstained with hematoxylin. TUNEL labeling was performed using the ApopTag Peroxydase In Situ Apoptosis detection kit (Millipore-S7100). Apoptotic indices were determined by counting the total number of TUNEL-positive cells or the number of TUNEL positive germ cells at different stages (Russell et al., 1990). Approximately 500 tubules were counted on at least 4 cross-sections located at midtestis for each animal.

### Gene expression studies

RNA was purified from tissues, primary Leydig cells or cultured cells using TRIZOL (Invitrogen). RNA isolation, cDNA preparation and qPCR analysis was carried out following standard protocols. qPCR analyses were performed using specific quantitative PCR primers from SABiosciences (<http://www.sabiosciences.com/RT2PCR.php>).

### cAMP quantification

For cAMP measurements TM3 Leydig cells were plated in 6 cm dishes ( $10^7$  cells per dish) 1 day before the experiment. Cells were serum starved for 16 h (in the presence of 0.1% BSA), then pre-incubated in the presence of 0.5 mM IBMX for 30 min and stimulated with indicated concentration of osteocalcin also in the presence of 0.5 mM IBMX for 30 min. cAMP concentration were measured with the Parameter cAMP kit (R&D Systems, KGE002).

### Receptor binding assays

For binding studies, testes from 8 week-old mice were snap frozen in liquid nitrogen, and 20  $\mu$ m thick sections were prepared and desiccated overnight at 4°C under vacuum. On the following day, sections were rehydrated in ice-cold binding buffer (50 mM TrisHCl, pH 7.4, 10 mM MgCl<sub>2</sub>, 0.1 mM EDTA and 0.1% BSA) for 15 min and incubated for 1 h in the presence of biotinylated osteocalcin. For competition assays, a 100-fold molar excess of unlabeled osteocalcin, glycine, lysine or hCG was added. After 3 washes in cold PBS, sections were incubated for 1 h in the detection system containing 0.1% BSA (ABC Elite, Vector Laboratories), washed again and incubated with DAB peroxidase substrate kit (Vector Laboratories) according to the manufacturer's protocol. After a final wash, sections were mounted in water-based mounting medium. As negative controls we used sections incubated with the detection system only (ABC Elite and DAB) or *Gprc6a*<sup>-/-</sup> testis sections (Basura et al., 2008).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

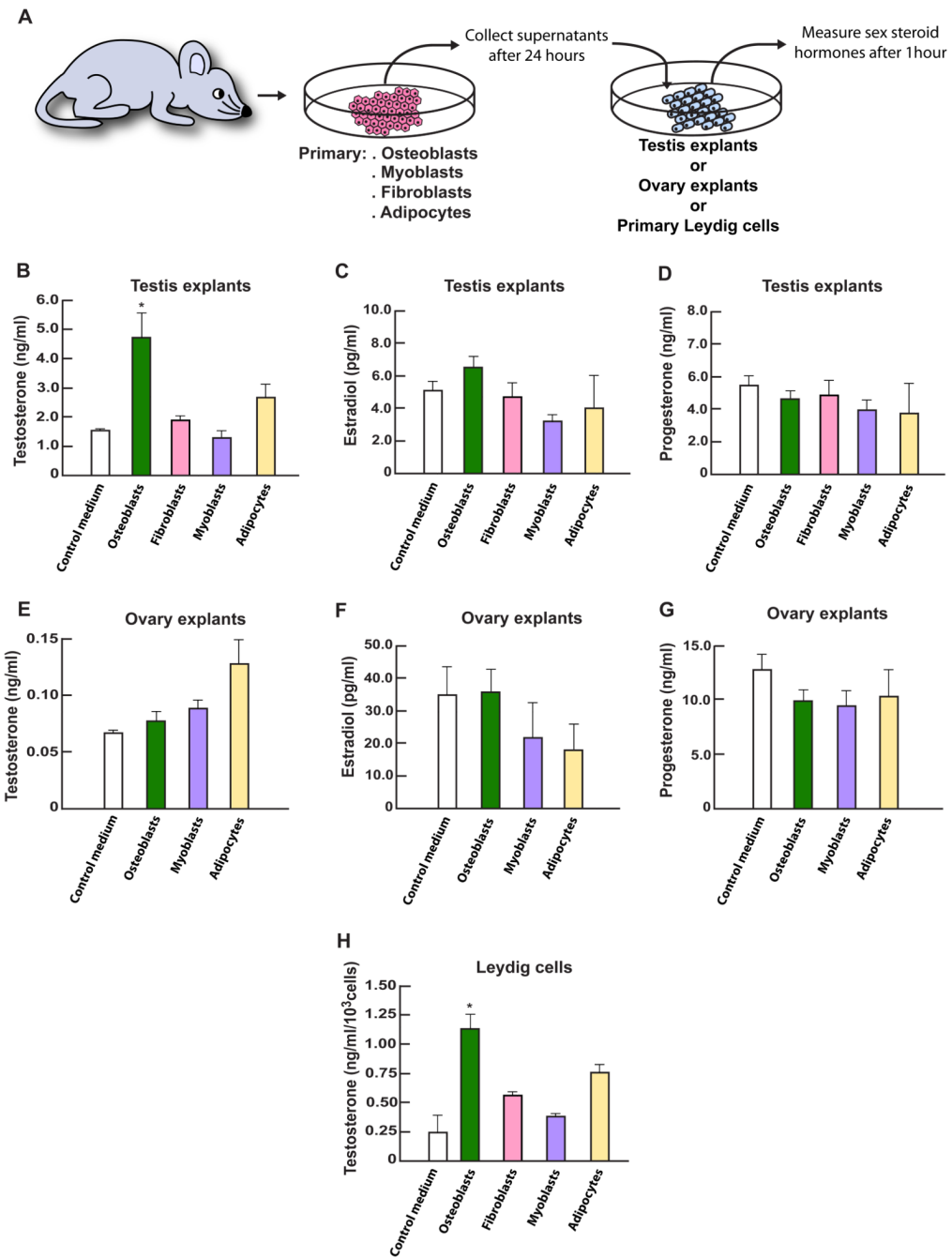
We thank Drs. Behringer and Ko for generously providing the *Amh-mis-Cre* and *Cyp17-iCre* mice, Dr. Hampson for generously providing us Gprc6a antibody, Dr. AF Parlow for measurements of LH in serum (National Hormone and Peptide Program), Drs. Przedborski and Jackson-Lewis for gracious help with stereological analysis, Drs. Mark, Yadav, Yoshizawa, Allen and Setola for critical experimental advice and G. Ren for mouse genotyping. This work was supported by grants from the NIH (GK), Fond de la recherche en santé du Québec (MF) and HFSP (GS, FO).

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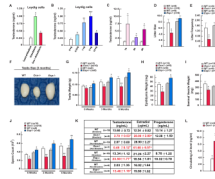
**Figure 1. Osteoblasts enhance testosterone biosynthesis by Leydig cells**

(A) Schematic representation of the cell-based assay used to determine the role of various mesenchymal cells in sex steroid hormone production. Various primary mesenchymal cells from mice were cultured in Leydig cell medium and supernatants were collected after 24 h. Then, testis or ovary explants or primary Leydig cells were cultured for 1 h with these supernatants and radioimmunoassays (RIAs) were performed to measure levels of testosterone, estradiol or progesterone.

(B–D) Testis explants cultured in the presence of supernatants of different mesenchymal cell cultures: RIA measurement of (B) testosterone, (C) estradiol and (D) progesterone levels.



**(E–G)** Ovary explants cultured in presence of supernatants of different mesenchymal cells cultures: RIA measurement of **(E)** testosterone, **(F)** estradiol and **(G)** progesterone levels. **(H)** Testosterone production by primary Leydig cells cultured in the presence of supernatants of different mesenchymal cell cultures. Error bars represent SEM. Student's t test (\*)  $P < 0.05$ .



**Figure 2. Osteocalcin favors male fertility by increasing testosterone production by Leydig cells**

(A) Testosterone production by primary Leydig cells cultured in the presence of supernatants of wild type (WT) or *Ocn*<sup>-/-</sup> osteoblast cultures.

(B) Testosterone production by primary Leydig cells following stimulation with increasing doses of osteocalcin.

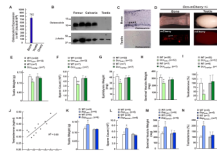
(C) Circulating testosterone levels in WT mice 1 h, 4 h and 8 h after vehicle or osteocalcin injection (3 ng/g of body weight).

(D–E) Comparison between the average litter size (D) and frequency (E) generated by WT, *Ocn*<sup>-/-</sup> or *Esp*<sup>-/-</sup> male littermate mice crossed with WT females (breeding was tested from 8 to 16 weeks of age).

(F–J) Testis size (F), testis weight (G), epididymis weight (H), seminal vesicle weights (I) and sperm counts (J) in *Ocn*<sup>-/-</sup> and *Esp*<sup>-/-</sup> compared to WT non-breeder littermate mice.

(K) Circulating sex steroid levels in *Ocn*<sup>-/-</sup> and *Esp*<sup>-/-</sup> compared to WT littermate mice. The analyses were performed on breeders and non-breeder mice.

(L) Circulating LH levels in *Ocn*<sup>-/-</sup> compared to WT non-breeder littermate mice. Error bars represent SEM. Student's t test (\*) P<0.05, (\*\*) P<0.001.



**Figure 3. Osteocalcin promotes male fertility through its expression in osteoblasts**

(A) qPCR analysis of *Osteocalcin* expression in bone, testes and ovaries of 3 month-old non-breeder WT mice.

(B) Western blot analysis of osteocalcin in femur, calvaria and testis.

(C) *In situ* hybridization analysis of *Osteocalcin* expression in bone and testis of 3 month-old WT mice.

(D) Analysis of mCherry fluorescent protein in bone and testis of *Osteocalcin-mCherry* knock-in mice.

(E–H) Fertility in mice lacking *Ocn* specifically in osteoblasts (*Ocn<sub>osb</sub><sup>-/-</sup>*) or Leydig cells (*Ocn<sub>Leydig</sub><sup>-/-</sup>*) compared to WT non-breeder littermates: (E) testes weight, (F) sperm count, (G) epididymis and (H) seminal vesicle weights.

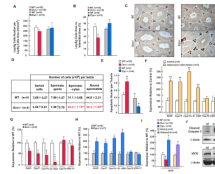
(I) Ratio of circulating testosterone levels measured in WT and *Ocn<sub>osb</sub><sup>-/-</sup>* or in WT and *Ocn<sub>Leydig</sub><sup>-/-</sup>* non-breeder littermate mice.

(J) Linear regression representation of circulating testosterone levels versus circulating osteocalcin levels in *Ocn<sub>osb</sub><sup>-/-</sup>* (n=11) non-breeder mice. Each dot represents one *Ocn<sub>osb</sub><sup>-/-</sup>* mouse. In WT littermate mice, the levels of osteocalcin varied from 106 to 177 ng/ml (on average, 133 ng/ml). For *Ocn<sub>osb</sub><sup>-/-</sup>* the average osteocalcin level was 68.4 ng/ml.

(K–M) Fertility in mice lacking *Esp* specifically in osteoblasts (*Esp<sub>osb</sub><sup>-/-</sup>*) or Leydig cells (*Esp<sub>Leydig</sub><sup>-/-</sup>*) compared to WT non-breeder littermates: (K) Testis weight, (L) sperm count and (M) seminal vesicle weight.

(N) Ratio of circulating testosterone levels measured in WT and *Esp<sub>osb</sub><sup>-/-</sup>* or in WT and *Esp<sub>Leydig</sub><sup>-/-</sup>* non-breeder littermate mice.

Error bars represent SEM. Student's t test (\*) P<0.05, (\*\*) P<0.001.



**Figure 4. Cellular and molecular events triggered by osteocalcin in Leydig cells**

(A–C) Histological analyses of Leydig cells in *Ocn*<sup>-/-</sup> and *Esp*<sup>-/-</sup> non-breeder mice (A)

Absolute number of Leydig cells per testis was quantified by the number of 3β-HSD positive cells. (B) Ratio between Leydig cells (immunopositive for 3β-HSD) versus testis interstitial areas in WT, *Ocn*<sup>-/-</sup> and *Esp*<sup>-/-</sup> non-breeder mice. (C) 3β-HSD

immunohistochemistry staining of WT, *Ocn*<sup>-/-</sup> and *Esp*<sup>-/-</sup> testes.

(D) Quantification of the different testicular cell types in WT and *Ocn*<sup>-/-</sup> non-breeder mice.

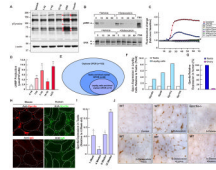
(E) Germ cell apoptosis analysis by TUNEL assay in WT, *Ocn*<sup>-/-</sup> and *Esp*<sup>-/-</sup> non-breeder testes.

(F–H) qPCR analysis of the expression of steroidogenic acute regulatory protein (*StAR*), cholesterol side-chain cleavage enzyme (*Cyp11a*), cytochrome P-450 17 alpha (*Cyp17*), 3-β-hydroxysteroid dehydrogenase (*3β-HSD*), aromatase enzyme (*Cyp19*) and 17-β-hydroxysteroid dehydrogenase (*HSD-17*) in primary Leydig cells treated with 3 ng/ml of osteocalcin (F), in *Ocn*<sup>-/-</sup> compared to WT non-breeder littermate testes (G) and in *Esp*<sup>-/-</sup> compared to WT non-breeder littermate testes (H).

(I) qPCR analysis of *Grth/Ddx25* expression in WT, *Ocn*<sup>-/-</sup>, *Esp*<sup>-/-</sup> and WT non-breeder mice treated with vehicle or osteocalcin (3 ng/g of body weight).

(J) Western blot analysis of cleaved caspase 3 and tACE in WT and *Ocn*<sup>-/-</sup> non-breeder testes.

Error bars represent SEM. Student's t test (\*) P<0.05, (\*\*) P<0.001.



**Figure 5. G-protein coupled receptor Gprc6a is a receptor for osteocalcin**

(A) Anti-phospho-tyrosine antibody Western blot analysis of TM3 Leydig cells treated with increasing concentrations of osteocalcin, or 10% FBS or insulin as positive controls, for 1 min (upper panel). Proteins phosphorylated on tyrosine residues appear in positive controls (asterisks) but not in osteocalcin treated cells. Equal loading was assessed using an anti-actin antibody (lower panel).

(B) Western blot analysis of TM3 Leydig cells showing the absence of ERK1/2 phosphorylation upon stimulation with vehicle or osteocalcin.

(C) Calcium fluxes in primary Leydig cells upon stimulation with increasing doses of osteocalcin. 10% FBS and ionophore (A23187) were used as positive controls.

(D) cAMP production upon osteocalcin stimulation is increased in TM3 Leydig cells.

(E) Schematic representation of the results obtained by the differential expression search for osteocalcin receptors. Among the 103 orphan GPCRs expressed in testis and ovary 22 were predominantly expressed in testis and only four were enriched in primary Leydig cells compared to the expression in whole testis.

(F) Relative expression of *Gprc6a*, *Gpr45*, *Gpr112* and *Gpr139* in Leydig cells compared to whole testis.

(G) qPCR analysis of *Gprc6a* expression in human testis and ovary.

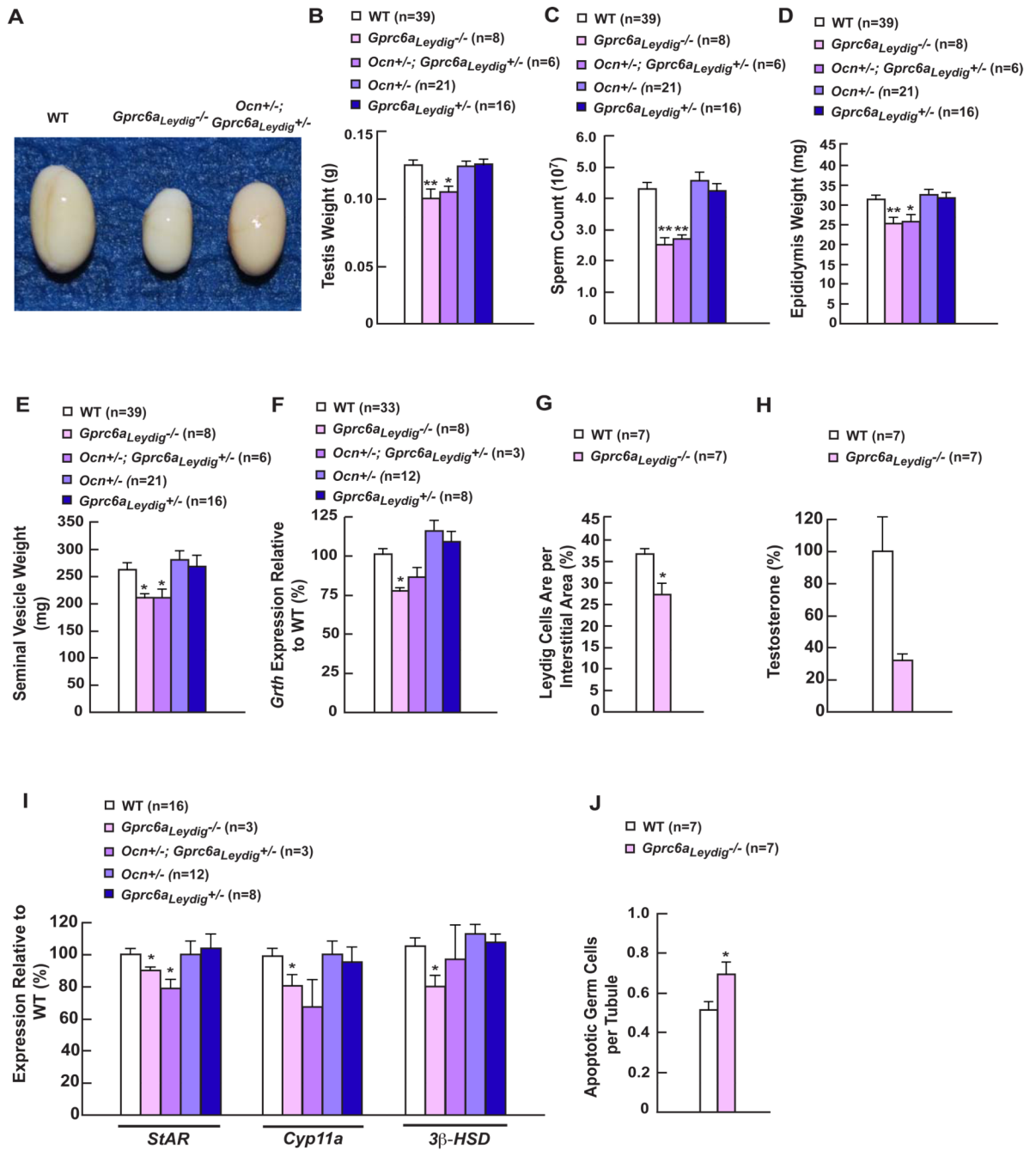
(H) Immunofluorescence analysis of Gprc6a expression in mice and human testis coronal sections. Anti-IgG was used as negative control.

(I) qPCR analysis of *Gprc6a* expression in 1, 4, 6 and 12 week-old WT testes.

(J) Cross sections of testes from WT and *Gprc6a*-deficient mice stained with biotinylated osteocalcin (b-osteocalcin). Upper left panel: WT testis stained with avidin-biotin complex only; upper middle panel: WT testis stained with 10 nM of b-osteocalcin; upper right panel: testis from *Gprc6a*-deficient mice stained with 10 nM of b-osteocalcin; lower left panel: WT testis stained with 10 nM of b-osteocalcin in the presence of 1000 nM hCG; lower middle panel: WT testis stained with 10 nM of b-osteocalcin in the presence of 1000 nM lysine; lower right panel: WT testis stained with 10 nM of b-osteocalcin in the presence of 1000 nM of unlabeled osteocalcin.

Error bars represent SEM. Student's t test (\*)  $P < 0.05$ , (\*\*)  $P < 0.001$ .





**Figure 6. Specific deletion of *Gprc6a* in Leydig cells decreases male fertility**

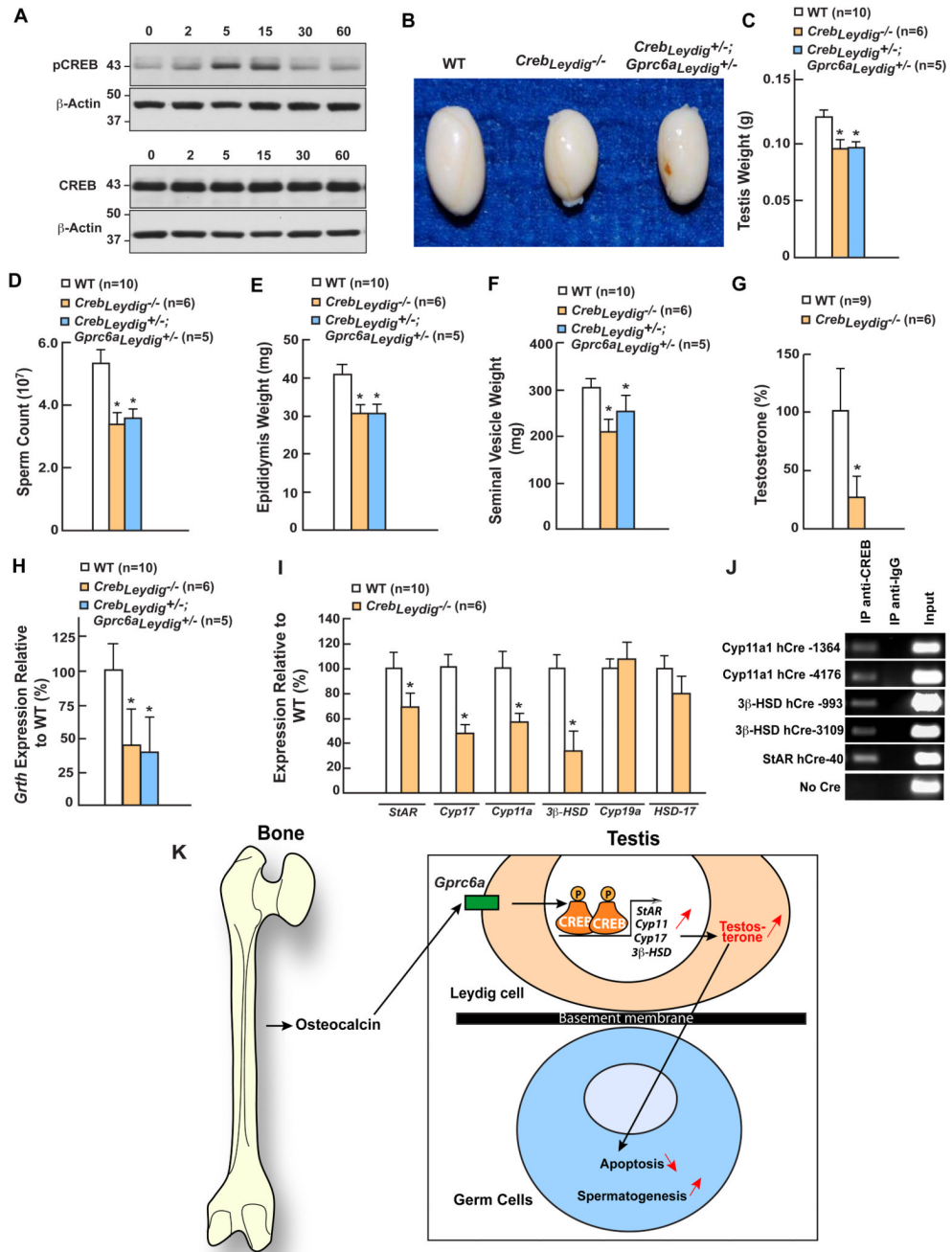
(A–E) Fertility in mice lacking *Gprc6a* in Leydig cells only (*Gprc6a<sup>Leydig</sup><sup>-/-</sup>*) or lacking one allele of *Ocn* or one allele of *Gprc6a* in Leydig cells only (*Ocn<sup>+/-</sup>* or *Gprc6a<sup>Leydig</sup><sup>+/-</sup>*), or in compound heterozygous mice (*Ocn<sup>+/-</sup>; Gprc6a<sup>Leydig</sup><sup>+/-</sup>*) compared to control littermates. (A) Testis size, (B) testis weight, (C) sperm count, (D–E) epididymis and seminal vesicle weights.

(F) qPCR analysis of *Grth* expression in mice of indicated genotypes.

(G) Ratio between Leydig cells (stained by immunohistochemistry of  $\beta$ -HSD) versus testis interstitial areas.

(H) Ratio of circulating testosterone levels measured in WT and *Gprc6a<sup>Leydig</sup><sup>-/-</sup>* mice.

**(I)** qPCR analysis of *StAR*, *Cyp11a*, *3 $\beta$ -HSD* in *Gprc6a<sub>Leydig</sub><sup>-/-</sup>* and *Ocn<sup>+/-</sup>*; *Gprc6a<sub>Leydig</sub><sup>+/-</sup>* compared to WT littermate testes.  
**(J)** Germ cell apoptosis analysis by TUNEL assay.  
All the analyses were performed in non-breeder mice.  
Error bars represent SEM. Student's t test (\*) P<0.05, (\*\*) P<0.001.



**Figure 7. CREB is a transcription factor mediating osteocalcin-evoked gene expression in Leydig cells**

(A) Western blot analysis of CREB activation upon stimulation with osteocalcin.

(B–F) Fertility in mice lacking *Creb* in Leydig cells (*CrebLeydig*<sup>-/-</sup>) or of compound heterozygous mice (*CrebLeydig*<sup>+/-</sup>; *Gprc6aLeydig*<sup>+/-</sup>) compared to control littermates. (B) Testis size, (C) testis weight, (D) sperm count, (E–F) epididymis and seminal vesicle weights.

(G) Quantification of circulating testosterone levels represented as fold change compared to WT.

(H) qPCR analysis of *Grth* expression in mice of indicated genotypes.

**(I)** qPCR analysis of *StAR*, *Cyp11a*, *Cyp17*, *3 $\beta$ -HSD*, *Cyp19* and *HSD-17* in *Creb<sub>Leydig</sub><sup>-/-</sup>* compared to control littermate testes.

**(J)** Chromatin immunoprecipitation (ChIP) using anti-CREB antibody and unspecific isotype IgG antibody in the TM3 cell line.

**(K)** Model representing current knowledge about the regulation of male fertility by the skeleton.

All the analyses were performed in non-breeders mice.

Error bars represent SEM. Student's t test (\*)  $P < 0.05$ .