Cell

Endocrine Regulation of Male Fertility by the Skeleton

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SUMMARY

Interactions between bone and the reproductive system have until now been thought to be limited to the regulation of bone remodeling by the gonads. We now show that, in males, bone acts as a regulator of fertility. Using coculture assays, we demonstrate that osteoblasts are able to induce testosterone production by the testes, though 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 that is 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 to hereafter 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 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 postmenopausal 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 10 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 functions of bone are mediated by an osteoblast-specific secreted molecule, osteocalcin, that, when uncarboxylated, acts as a hormone favoring β 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 that is 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 (>3-fold) but did not affect estradiol and progesterone secretion by testes or ovaries (Figures 1B–1G). In subsequent experiments, testes explants were replaced by defined cell populations. Because 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 type cultures. In the conditions of this assay, supernatants of osteoblast cultures were also the only ones that were able to significantly increase testosterone production by Leydig cells (>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 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 ($Ocn^{-/-}$) breed poorly (P.D. and G.K., unpublished data), 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 that is the case. First, supernatants of wild-type (WT), but not of $Ocn^{-/-}$, osteoblast cultures increased testosterone production by Leydig cells (Figure 2A). Second, treating Leydig cells with an 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- ($Ocn^{-/-}$ mice) and gain-of-function ($Esp^{-/-}$ mice) mouse models for osteocalcin (Lee et al., 2007). When $Ocn^{-/-}$ males were crossed with WT female mice, the size of the litters was nearly 2-fold smaller than when WT males were crossed with WT females (Figure 2D). Conversely, the number of pups per litter was increased when $Esp^{-/-}$ males were bred with WT female mice, although this increase did not reach statistical significance (Figure 2D). The frequency of litters for 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). Testes size and weight were significantly decreased in $Ocn^{-/-}$ and increased in $Esp^{-/-}$ mice at 3 months of age. In some of the latter mutant mice, this was caused, in part, by fluid accumulation (Figures 2F and 2G). The weights of

epididymides and seminal vesicles and sperm count were also significantly decreased in $Ocn^{-/-}$ and increased in $Esp^{-/-}$ mice (Figures 2H–2J). These abnormalities worsened over time (Figures 2G and 2J).

Motility of sperm from both WT and $Ocn^{-/-}$ males was assessed by videomicroscopy immediately after dissemination from the caudal epididymis or after 2 hr of incubation under conditions known to prepare sperm for fertilization (Suárez and Osman, 1987). In both cases, the percentage of motile sperm did not differ between $Ocn^{-/-}$ and WT mice (Figure S1A available online). Likewise, the percentage of abnormally shaped or dead sperm was similar in WT and $Ocn^{-/-}$ mice (Figures S1B and S1C).

Consistent with the fact that osteocalcin favors testosterone synthesis in Leydig cells ex vivo, circulating levels of testosterone were markedly decreased in $Ocn^{-/-}$ and increased in $Esp^{-/-}$ 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 $Ocn^{-/-}$ 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 $Ocn^{-/-}$ and WT mice, and although circulating levels of estradiol were higher in $Ocn^{-/-}$ than in WT mice, they remained within the normal range (from 9.3 to 28.9 ng/ml for nonbreeder mice and from 14.4 to 71.1 ng/ml for breeder mice) (Figure 2K). Estradiol levels were not affected in $Esp^{-/-}$ mice. As predicted by the coculture assays, female fertility, ovary weight, morphology of the uterus, follicles number, and circulating levels of sex steroid hormones were normal in $Ocn^{-/-}$ female mice (Figures S1D–S1L).

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 (Figures 3A–3C). To be able to trace *Osteocalcin*-expressing cells in vivo, we knocked the m*Cherry* fluorescent reporter gene into the *Ocn* locus (*Ocn-mCherry* mice) (Figures S2A and S2B). Though we observed the expected strong signal in osteoblasts, there was no detectable m*Cherry* 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* (Figures S2C and S2D) or *Esp* with either the $\alpha 1(I)$ *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
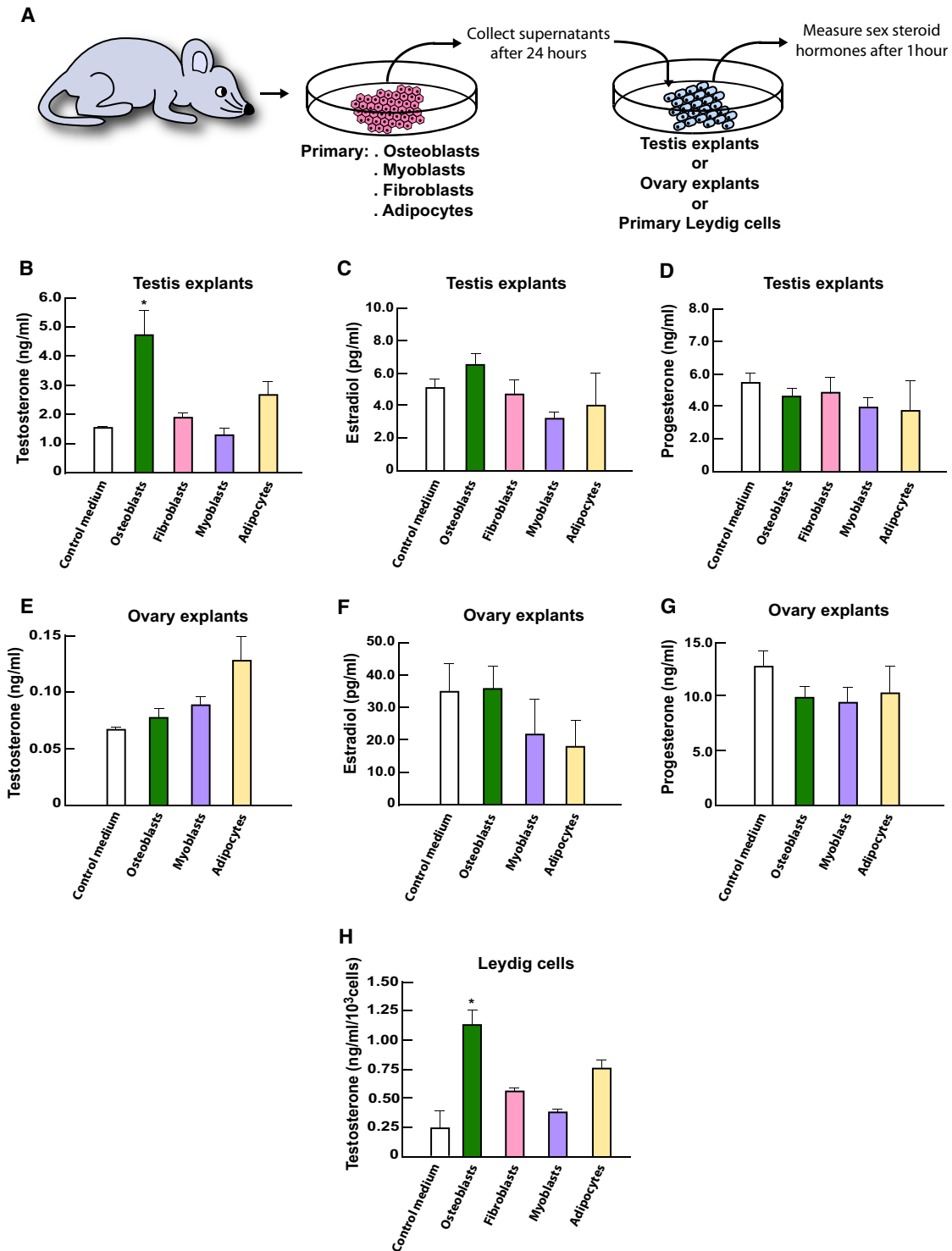
Figure 1. Osteoblasts Enhance Testosterone Biosynthesis by Leydig Cells

(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 hr. Then, testis or ovary explants or primary Leydig cells were cultured for 1 hr 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.
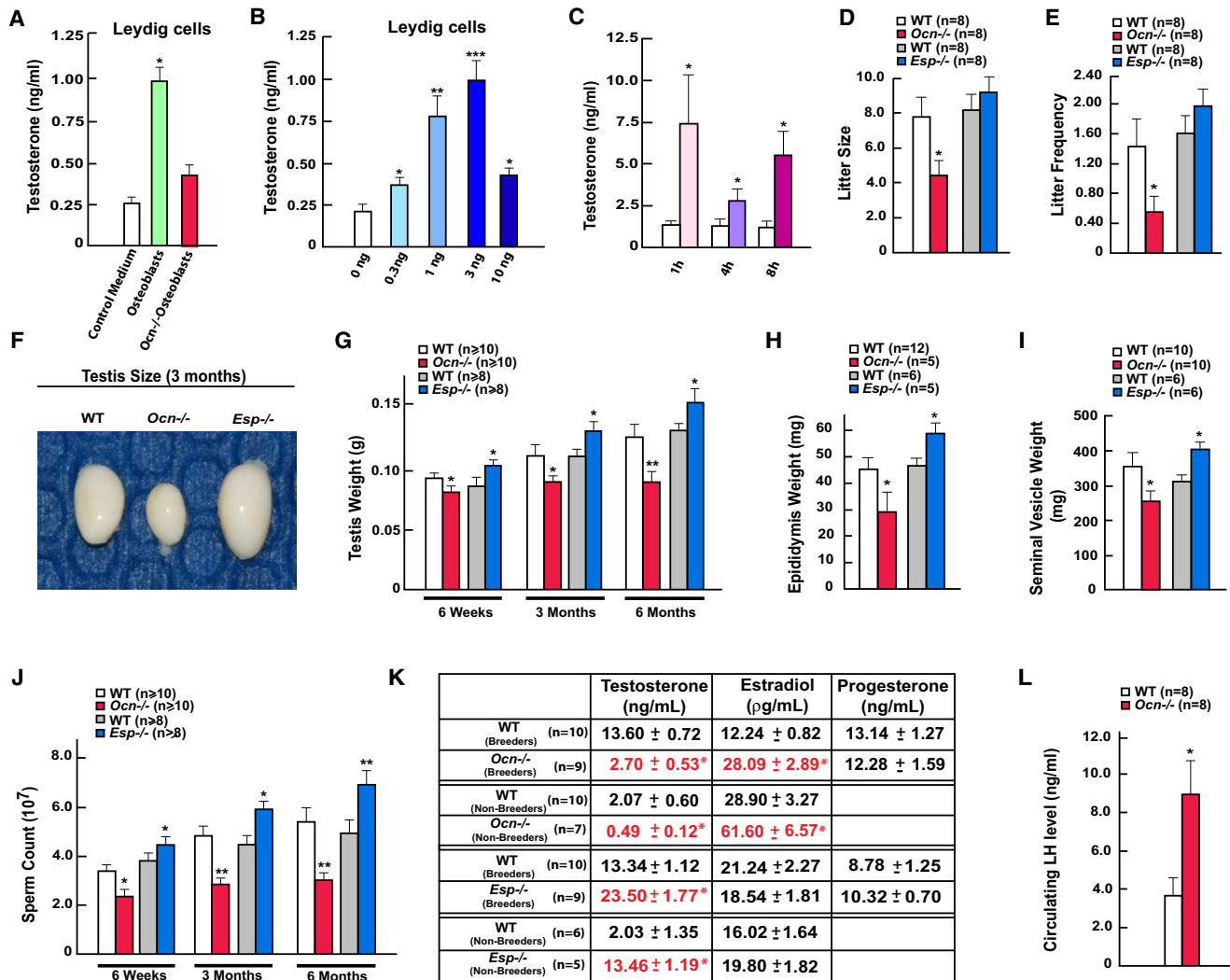
Cell

A Leydig cells — Testosterone (ng/ml): Control Medium, Osteoblasts*, Ocn-/-Osteoblasts

B Leydig cells — Testosterone (ng/ml): 0 ng, 0.3ng*, 1 ng**, 3 ng***, 10 ng*

C Testosterone (ng/ml): 1h*, 4h*, 8h*

D Litter Size — WT (n=8), Ocn-/- (n=8)*, WT (n=8), Esp-/- (n=8)

E Litter Frequency — WT (n=8), Ocn-/- (n=8)*, WT (n=8), Esp-/- (n=8)

F Testis Size (3 months): WT, Ocn-/-, Esp-/-

G Testis Weight (g) — WT (n≥10), Ocn-/- (n≥10), WT (n≥8), Esp-/- (n≥8): 6 Weeks, 3 Months, 6 Months

H Epididymis Weight (mg) — WT (n=12), Ocn-/- (n=5), WT (n=6), Esp-/- (n=5)

I Seminal Vesicle Weight (mg) — WT (n=10), Ocn-/- (n=10), WT (n=6), Esp-/- (n=6)

J Sperm Count (10^7) — WT (n≥10), Ocn-/- (n≥10), WT (n≥8), Esp-/- (n≥8): 6 Weeks, 3 Months, 6 Months

K

	Testosterone (ng/mL)	Estradiol (ρg/mL)	Progesterone (ng/mL)
WT (Breeders) (n=10)	13.60 ± 0.72	12.24 ± 0.82	13.14 ± 1.27
Ocn-/- (Breeders) (n=9)	2.70 ± 0.53*	28.09 ± 2.89*	12.28 ± 1.59
WT (Non-Breeders) (n=10)	2.07 ± 0.60	28.90 ± 3.27	
Ocn-/- (Non-Breeders) (n=7)	0.49 ± 0.12*	61.60 ± 6.57*	
WT (Breeders) (n=10)	13.34 ± 1.12	21.24 ± 2.27	8.78 ± 1.25
Esp-/- (Breeders) (n=9)	23.50 ± 1.77*	18.54 ± 1.81	10.32 ± 0.70
WT (Non-Breeders) (n=6)	2.03 ± 1.35	16.02 ± 1.64	
Esp-/- (Non-Breeders) (n=5)	13.46 ± 1.19*	19.80 ± 1.82	

L Circulating LH level (ng/ml) — WT (n=8), Ocn-/- (n=8)*

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 $Ocn^{-/-}$ osteoblast cultures.
(B) Testosterone production by primary Leydig cells following stimulation with increasing doses of osteocalcin.
(C) Circulating testosterone levels in WT mice 1 hr, 4 hr, and 8 hr after vehicle or osteocalcin injection (3 ng/g of body weight).
(D and E) Comparison between the average litter size (D) and frequency (E) generated by WT, $Ocn^{-/-}$, or $Esp^{-/-}$ 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 $Ocn^{-/-}$ and $Esp^{-/-}$ compared to WT nonbreeder littermate mice.
(K) Circulating sex steroid levels in $Ocn^{-/-}$ and $Esp^{-/-}$ compared to WT littermate mice. The analyses were performed on breeder and nonbreeder mice.
(L) Circulating LH levels in $Ocn^{-/-}$ compared to WT nonbreeder littermate mice.
Error bars represent SEM. Student's t test; *p < 0.05; **p < 0.001. See also Figure S1.

reduced in 12-week-old $Ocn_{osb}^{-/-}$ mice. None of these parameters were affected in mice lacking *Osteocalcin* in Leydig cells only (Figures 3E–3I). There was a tight correlation between circulating levels of osteocalcin and testosterone in $Ocn_{osb}^{-/-}$ mice (Figure 3J). Conversely, $Esp_{osb}^{-/-}$ mice displayed testicular abnormalities identical to those of $Esp^{-/-}$ mice. Inactivation of *Esp* in Sertoli cells, where this gene is expressed (Dacquin et al., 2004; Jamin et al., 2003), had no detectable deleterious

(E–G) Ovary explants cultured in the 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.
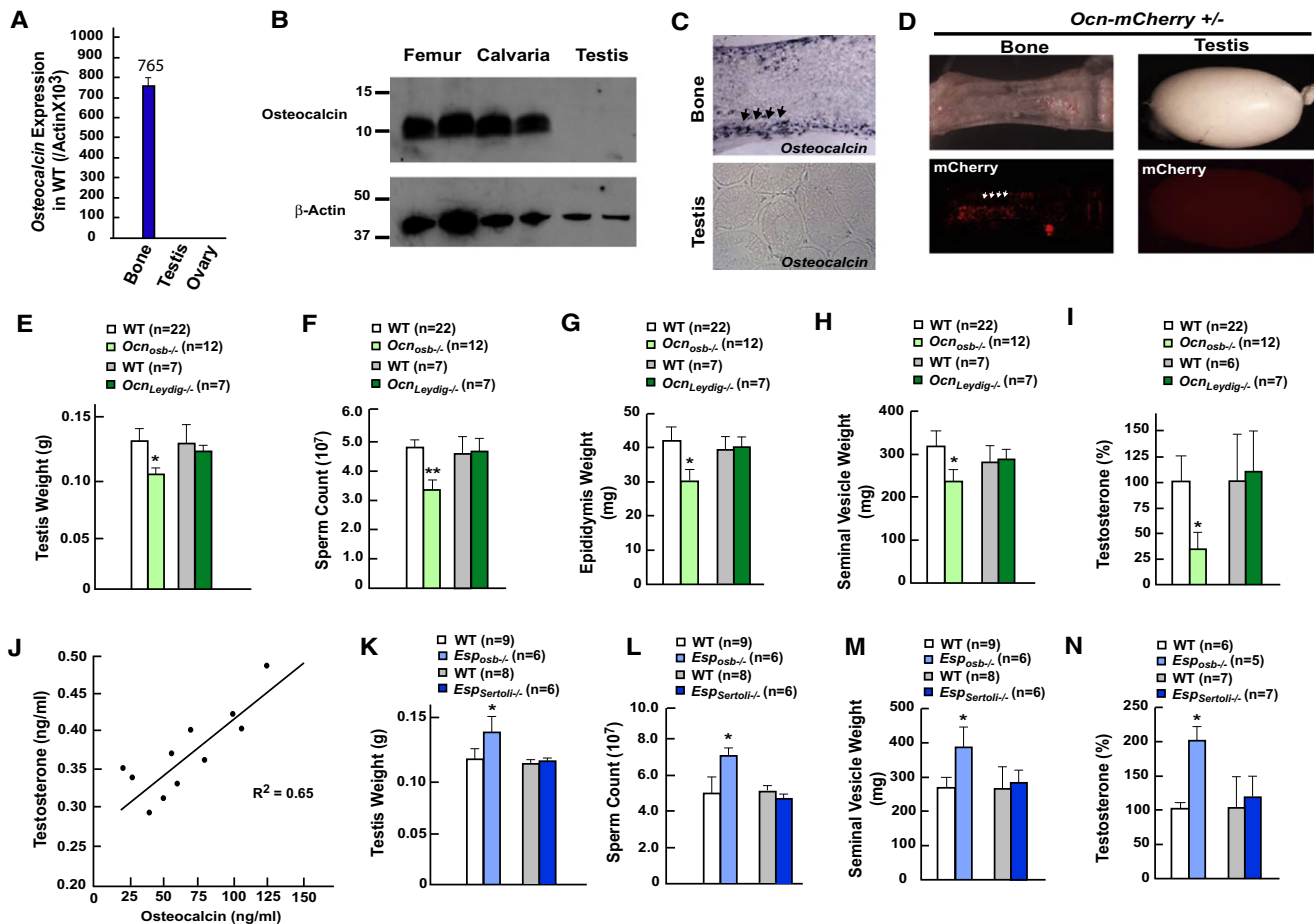Error bars represent SEM. Student's t test; *p < 0.05.

Figure 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 nonbreeder 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. Arrows show the *Osteocalcin* expression in bone.

(D) Analysis of mCherry fluorescent protein in bone and testis of *Osteocalcin-mCherry* knockin mice. Arrows show the presence of mCherry fluorescent protein reflecting *Osteocalcin*.

(E–H) Fertility in mice lacking *Ocn* specifically in osteoblasts ($Ocn_{osb}^{-/-}$) or Leydig cells ($Ocn_{Leydig}^{-/-}$) compared to WT nonbreeder littermates: (E) testes weight, (F) sperm count, (G) epididymis, and (H) seminal vesicle weights.

(I) Ratio of circulating testosterone levels measured in WT and $Ocn_{osb}^{-/-}$ or in WT and $Ocn_{Leydig}^{-/-}$ nonbreeder littermate mice.

(J) Linear regression representation of circulating testosterone levels versus circulating osteocalcin levels in $Ocn_{osb}^{-/-}$ (n = 11) nonbreeder mice. Each dot represents one $Ocn_{osb}^{-/-}$ mouse. In WT littermate mice, the levels of osteocalcin varied from 106 to 177 ng/ml (on average, 133 ng/ml). For $Ocn_{osb}^{-/-}$, the average osteocalcin level was 68.4 ng/ml.

(K–M) Fertility in mice lacking *Esp* specifically in osteoblasts ($Esp_{osb}^{-/-}$) or Leydig cells ($Esp_{Leydig}^{-/-}$) compared to WT nonbreeder littermates: (K) testis weight, (L) sperm count, and (M) seminal vesicle weight.

(N) Ratio of circulating testosterone levels measured in WT and $Esp_{osb}^{-/-}$ or in WT and $Esp_{Leydig}^{-/-}$ nonbreeder littermate mice.

Error bars represent SEM. Student's t test; *$p < 0.05$; **$p < 0.001$. See also Figure S2.

consequence on testis biology, demonstrating that it is through its expression in osteoblasts, not in Sertoli cells, that *Esp* regulates male fertility (Figures 3K–3N). 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-β-hydroxyste-

roid 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 $Ocn^{-/-}$ testes, as determined by the significant decrease of the ratio between the Leydig cells and interstitial areas observed in $Ocn^{-/-}$ compared to WT testes (Figures 4B and 4C). Conversely, this ratio was increased in $Esp^{-/-}$ testes (Figures 4B and 4C).

When germ cells were analyzed through a stereological approach, we observed that the number of spermatocytes and
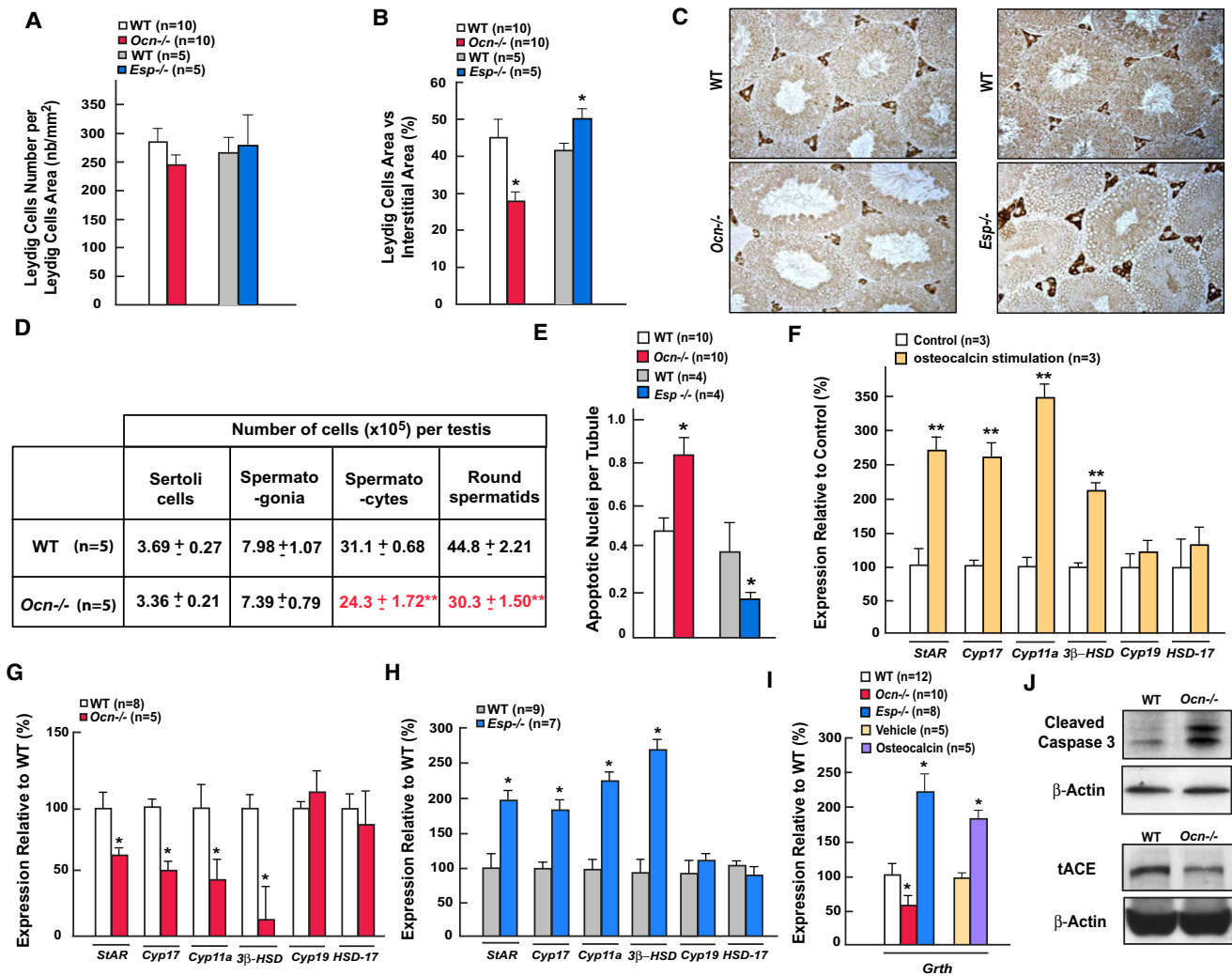
Figure 4. Cellular and Molecular Events Triggered by Osteocalcin in Leydig Cells

(A–C) Histological analyses of Leydig cells in $Ocn^{-/-}$ and $Esp^{-/-}$ nonbreeder 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, $Ocn^{-/-}$, and $Esp^{-/-}$ nonbreeder mice.
(C) 3β-HSD immunohistochemistry staining of WT, $Ocn^{-/-}$, and $Esp^{-/-}$ testes.
(D) Quantification of the different testicular cell types in WT and $Ocn^{-/-}$ nonbreeder mice.
(E) Germ cell apoptosis analysis by TUNEL assay in WT, $Ocn^{-/-}$, and $Esp^{-/-}$ nonbreeder testes.
(F–H) qPCR analysis of the expression of steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage enzyme (Cyp11a), cytochrome P-450 17 α (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 $Ocn^{-/-}$ compared to WT nonbreeder littermate testes (G), and in $Esp^{-/-}$ compared to WT nonbreeder littermate testes (H).
(I) qPCR analysis of Grth/Ddx25 expression in WT, $Ocn^{-/-}$, $Esp^{-/-}$, and WT nonbreeder 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 $Ocn^{-/-}$ nonbreeder testes.
Error bars represent SEM. Student's t test; *$p < 0.05$; **$p < 0.001$. See also Figure S3.

round spermatids was significantly decreased in $Ocn^{-/-}$ 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). Because testosterone inhibits germ cell apoptosis (Brinkworth et al., 1995; Henriksén et al., 1995; Sinha Hikim and Swerdloff, 1999), we performed TUNEL assays. Those

showed a 2-fold increase in germ cell apoptosis in $Ocn^{-/-}$ compared to WT mice and a 2-fold decrease in $Esp^{-/-}$ 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 $Ocn^{-/-}$ or $Esp^{-/-}$ mice

(Figure S3A). Taken together, these data further indicate that the decrease in fertility demonstrated by $Ocn^{-/-}$ 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 that are necessary for testosterone biosynthesis such as $StAR$, $Cyp11a$, $Cyp17$, and $3\beta\text{-}HSD$. In cell culture, uncarboxylated osteocalcin increased expression of these genes in Leydig cells (Figure 4F). Accordingly, their expression was significantly decreased in $Ocn^{-/-}$ and increased in $Esp^{-/-}$ testes (Figures 4G and 4H), whereas it was unaffected in $Ocn^{-/-}$ and $Esp^{-/-}$ ovaries or adrenal glands (Figures S3D–S3G). Of note, there was no change in expression of $Cyp19$, the gene encoding the testosterone aromatase, or of $HSD\text{-}17$ in $Ocn^{-/-}$ and $Esp^{-/-}$ testes (Figures 4F–4H).

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, 2010). $Grth$ expression was decreased in $Ocn^{-/-}$ and increased in $Esp^{-/-}$ 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 $Ocn^{-/-}$ 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 (Figures 5A–5D). 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 testes at a level at least 5-fold higher than in ovary. Twenty-two out of 103 orphan GPCRs tested were predominantly expressed in testes; of these 22, four were enriched in Leydig cells (Figures 5E and 5F). Among

them, $Gprc6a$ caught our attention because its deletion in all cells results in a metabolic and fertility phenotype similar to that of $Ocn^{-/-}$ 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 (Figures 5G and 5H and Figure S4B). Postnatally, $Gprc6a$ 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 $Gprc6a_{Leydig}^{-/-}$ mice. Prior to analyzing these $Gprc6a_{Leydig}^{-/-}$ mice, we verified that we had deleted $Gprc6a$, albeit partially (75% of deletion), in Leydig cells, but not in other organs (Figures S5C and S5D). In $Gprc6a_{Leydig}^{-/-}$ 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 three genes controlling testosterone biosynthesis that are regulated by osteocalcin (Figures 6A–6I 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 ($Ocn^{+/-};Gprc6a_{Leydig}^{+/-}$ mice). Whether we looked at testes, epididymis and seminal vesicle weights, or sperm count, $Ocn^{+/-};Gprc6a_{Leydig}^{+/-}$ mice had a phenotype identical to that observed in $Gprc6a_{Leydig}^{-/-}$ and $Ocn_{osb}^{-/-}$ mice (Figures 6A–6I).

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, we generated mice that lack CREB expression specifically in Leydig cells ($Creb_{Leydig}^{-/-}$ mice).

Twelve-week-old $Creb_{Leydig}^{-/-}$ 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 $Ocn^{-/-}$ and $Gprc6a_{Leydig}^{-/-}$ mice (Figures 7B–7G and Figure S6). $Creb_{Leydig}^{-/-}$ mice also
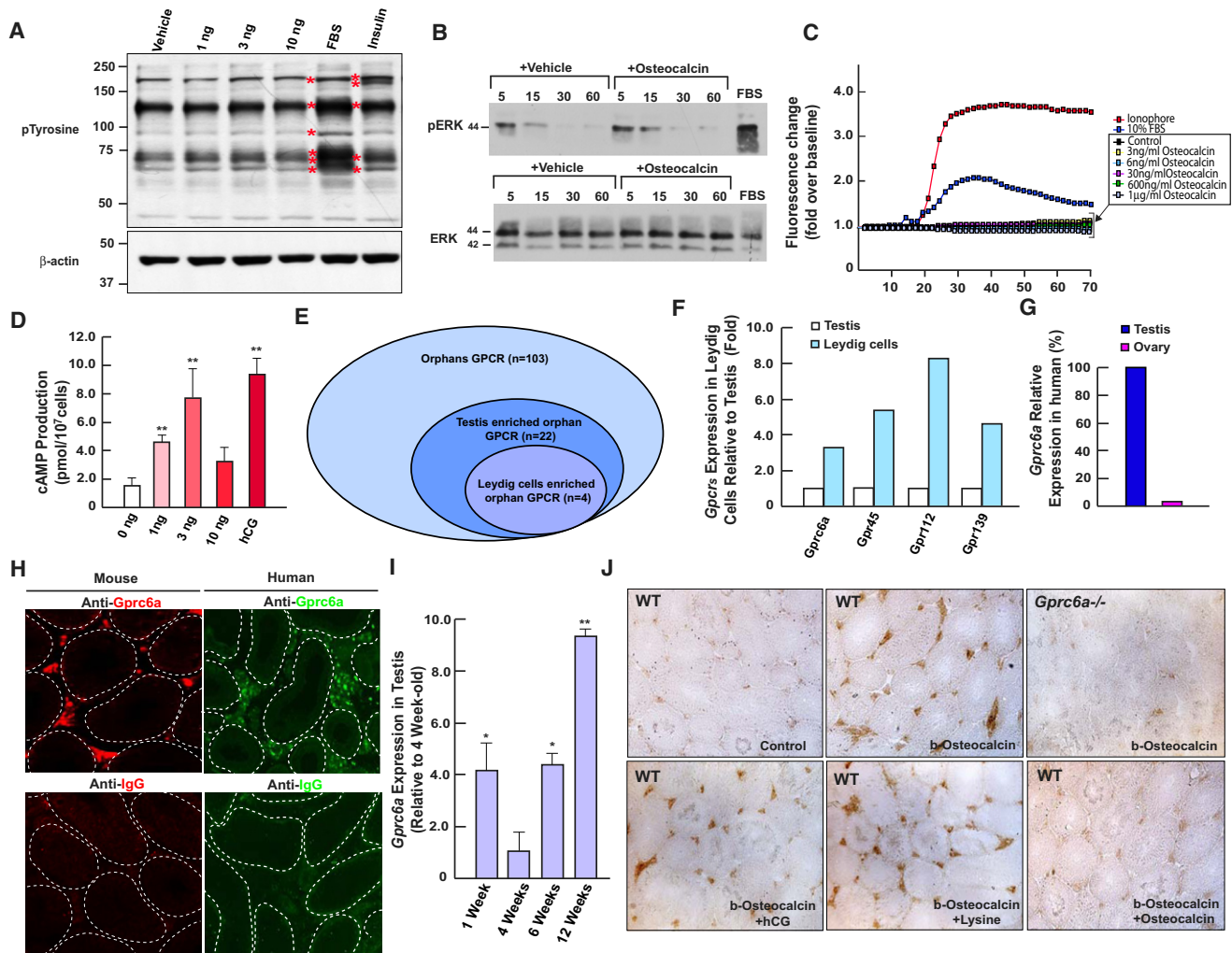
Figure 5. G Protein-Coupled Receptor Gprc6a Is a Receptor for Osteocalcin

(A) Anti-phosphotyrosine 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 (top). 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 (bottom).

(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) WT testis stained with avidin-biotin complex only. (Upper-middle) WT testis stained with 10 nM of b-osteocalcin. (Upper-right) Testis from *Gprc6a*-deficient mice stained with 10 nM of b-osteocalcin. (Lower-left) WT testis stained with 10 nM of b-osteocalcin in the presence of 1000 nM hCG. (Lower-middle) WT testis stained with 10 nM of b-osteocalcin in the presence of 1000 nM lysine. (Lower-right) 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. See also Figure S4.

demonstrated a strong decrease in the expression of *Grth* and of the four genes involved in testosterone biosynthesis whose expression is regulated by osteocalcin (Figures 7H and 7I). 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
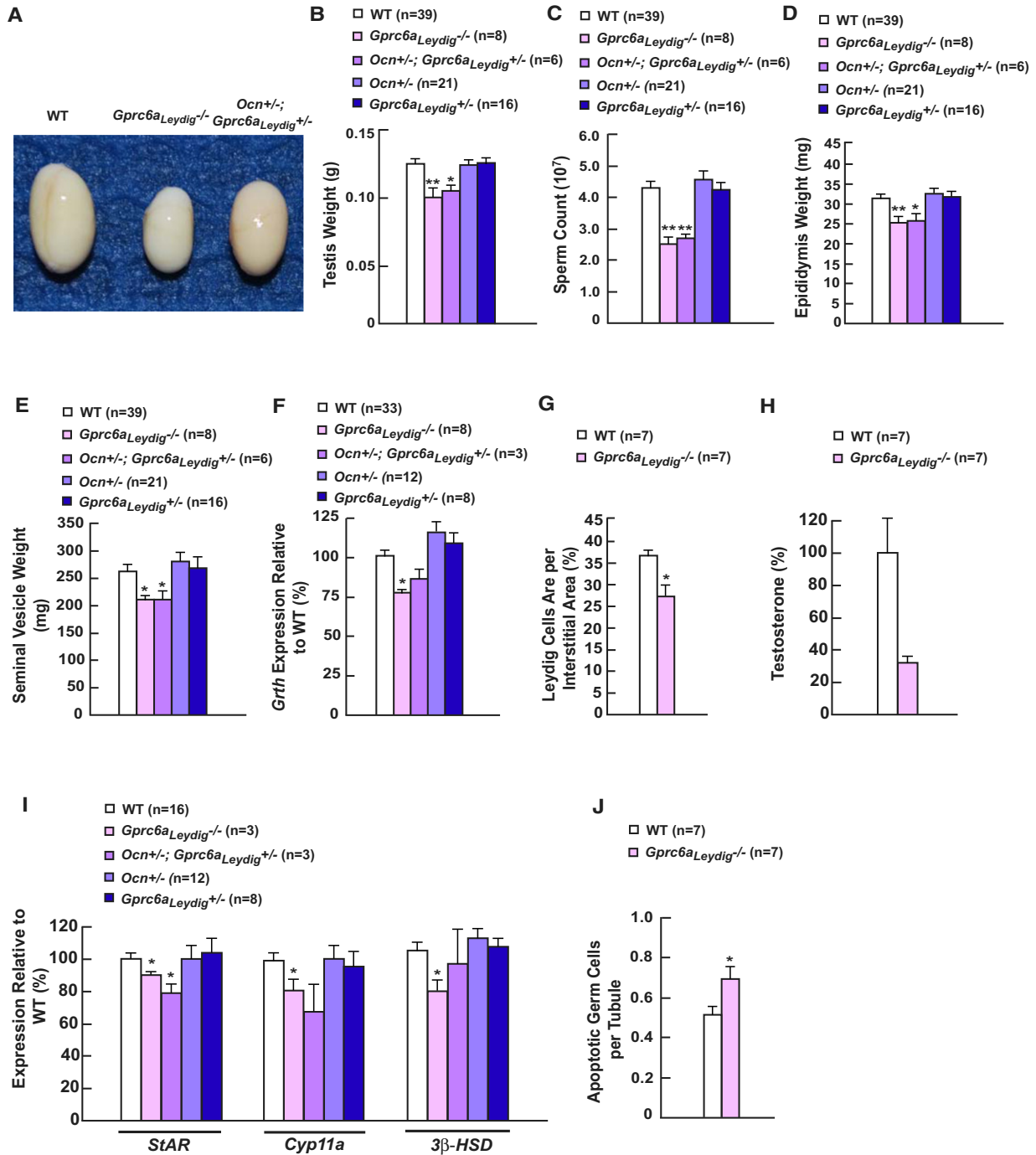
Figure 6. Specific Deletion of *Gprc6a* in Leydig Cells Decreases Male Fertility

(A–E) Fertility in mice lacking *Gprc6a* in Leydig cells only (*Gprc6a*$_{Leydig}^{-/-}$) or lacking one allele of *Ocn* or one allele of *Gprc6a* in Leydig cells only (*Ocn*$^{+/-}$ or *Gprc6a*$_{Leydig}^{+/-}$) or in compound heterozygous mice (*Ocn*$^{+/-}$; *Gprc6a*$_{Leydig}^{+/-}$) compared to control littermates. (A) Testis size, (B) testis weight, (C) sperm count, and (D and 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 3β-HSD) versus testis interstitial areas.

(H) Ratio of circulating testosterone levels measured in WT and *Gprc6a*$_{Leydig}^{-/-}$ mice.

(I) qPCR analysis of *StAR*, *Cyp11a*, and *3β-HSD* in *Gprc6a*$_{Leydig}^{-/-}$ and *Ocn*$^{+/-}$; *Gprc6a*$_{Leydig}^{+/-}$ compared to WT littermate testes.

(J) Germ cell apoptosis analysis by TUNEL assay.

All of the analyses were performed in nonbreeder mice. Error bars represent SEM. Student's t test; *p < 0.05; **p < 0.001. See also Figure S5.

Gprc6a in Leydig cells. The decrease in fertility demonstrated by *Creb*$_{Leydig}$$^{+/-}$;*Gprc6a*$_{Leydig}$$^{+/-}$ male mice was similar to that observed in *Creb*$_{Leydig}$$^{-/-}$ or *Gprc6a*$_{Leydig}$$^{-/-}$ male mice (Figures 7B–7F 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 that is present on Leydig cells of the testes 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 10 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. First, the dialog between bone physiology and energy metabolism and the paramount influence of the brain in the control of bone mass accrual became apparent (Karsenty, 2006). Second, 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 female, mice. Our observation that *Ocn*$^{-/-}$ 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 (Henriksén et al., 1995; Sinha Hikim and Swerdloff, 1999; Walker, 2009). Because 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 because germ cells do not express androgen receptor (Bremner et al., 1994; De Gendt et al., 2004; Wang et al., 2003). Unexpectedly, *Ocn*$^{-/-}$ 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*$^{-/-}$ 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 Bräuner-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., 2005, 2008, 2010). 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, though 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 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. First, and most importantly, this work
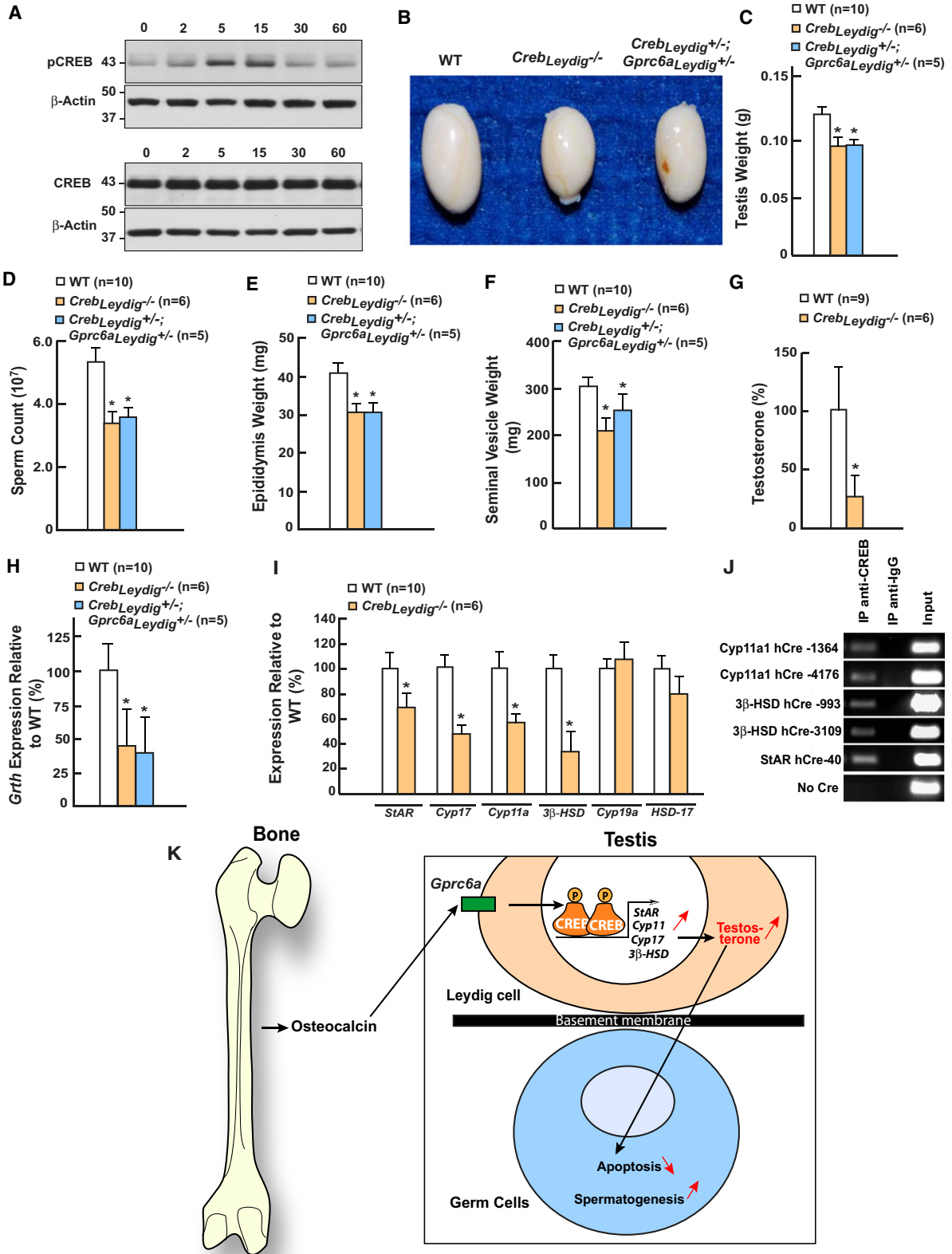
was, in part, initiated because of a clinical observation made in humans: loss of sex steroid hormones triggers a decrease in bone mass. This led us to test whether feedback regulation of fertility by bone may also occur. Second, *Gprc6a* is expressed, in human and in mice, in Leydig cells of the testes, 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* knockin, *Ocn* conditional, and *Gprc6a* conditional allele generation strategies are described in Figure S2 and Figure 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 1 × Pen-Strep, 25 mM HEPES (pH 7.4), and 0.07% BSA at 33°C in 5% CO_2. After 3 hr 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 three times with PBS and placed in serum-free RPMI medium for 2 hr before being used for experiments.

Osteocalcin Stimulation of Leydig Cells or Testes Explants
Primary Leydig cells and testes explants were washed three 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 hr, an aliquot of medium was collected for measurements of testosterone. Cells were then maintained for 3 additional hr 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 hr. 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 μm. For histological analysis, testes and ovaries sections were stained with periodic acid-Schiff and counterstained with hematoxylin. TUNEL labeling was performed using the ApopTag Peroxidase 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 four 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 hr (in the presence of 0.1% BSA) and then preincubated 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 was 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 μm 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 $MgCl_2$, 0.1 mM EDTA, and 0.1% BSA) for 15 min and incubated for 1 hr in the presence of biotinylated osteocalcin. For competition assays, a 100-fold molar excess of unlabeled osteocalcin, glycine, lysine, or hCG was added. After three washes in cold PBS, sections were incubated for 1 hr 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 $Gprc6a^{-/-}$ testis sections (Basura et al., 2008).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and six figures and can be found with this article online at doi:10.1016/j.cell.2011.02.004.

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 ($Creb_{Leydig}^{-/-}$) or of compound heterozygous mice ($Creb_{Leydig}^{+/-}$; $Gprc6a_{Leydig}^{+/-}$) compared to control littermates. (B) Testis size, (C) testis weight, (D) sperm count, and (E and 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*, *3β-HSD*, *Cyp19*, and *HSD-17* in $Creb_{Leydig}^{-/-}$ 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 of the analyses were performed in nonbreeder mice.
Error bars represent SEM. Student's t test; *p < 0.05. See also Figure S6.

REFERENCES

Basura, G.J., Abbas, A.I., O'Donohue, H., Lauder, J.M., Roth, B.L., Walker, P.D., and Manis, P.B. (2008). Ontogeny of serotonin and serotonin2A receptors in rat auditory cortex. Hear. Res. *244*, 45–50.

Bremner, W.J., Millar, M.R., Sharpe, R.M., and Saunders, P.T. (1994). Immunohistochemical localization of androgen receptors in the rat testis: evidence for stage-dependent expression and regulation by androgens. Endocrinology *135*, 1227–1234.

Bridges, P.J., Koo, Y., Kang, D.W., Hudgins-Spivey, S., Lan, Z.J., Xu, X., DeMayo, F., Cooney, A., and Ko, C. (2008). Generation of Cyp17iCre transgenic mice and their application to conditionally delete estrogen receptor alpha (Esr1) from the ovary and testis. Genesis *46*, 499–505.

Brinkworth, M.H., Weinbauer, G.F., Schlatt, S., and Nieschlag, E. (1995). Identification of male germ cells undergoing apoptosis in adult rats. J. Reprod. Fertil. *105*, 25–33.

Dacquin, R., Starbuck, M., Schinke, T., and Karsenty, G. (2002). Mouse alpha1 (I)-collagen promoter is the best known promoter to drive efficient Cre recombinase expression in osteoblast. Dev. Dyn. *224*, 245–251.

Dacquin, R., Mee, P.J., Kawaguchi, J., Olmsted-Davis, E.A., Gallagher, J.A., Nichols, J., Lee, K., Karsenty, G., and Smith, A. (2004). Knock-in of nuclear localised beta-galactosidase reveals that the tyrosine phosphatase Ptprv is specifically expressed in cells of the bone collar. Dev. Dyn. *229*, 826–834.

Dakhova, O., O'Day, D., Kinet, N., Yucer, N., Wiese, M., Shetty, G., and Ducy, P. (2009). Dickkopf-like1 regulates postpubertal spermatocyte apoptosis and testosterone production. Endocrinology *150*, 404–412.

De Gendt, K., Swinnen, J.V., Saunders, P.T., Schoonjans, L., Dewerchin, M., Devos, A., Tan, K., Atanassova, N., Claessens, F., Lécureuil, C., et al. (2004). A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. Proc. Natl. Acad. Sci. USA *101*, 1327–1332.

Ducy, P., Amling, M., Takeda, S., Priemel, M., Schilling, A.F., Beil, F.T., Shen, J., Vinson, C., Rueger, J.M., and Karsenty, G. (2000). Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. Cell *100*, 197–207.

Dufau, M.L., and Tsai-Morris, C.H. (2007). Gonadotropin-regulated testicular helicase (GRTH/DDX25): an essential regulator of spermatogenesis. Trends Endocrinol. Metab. *18*, 314–320.

Feldman, H.A., Longcope, C., Derby, C.A., Johannes, C.B., Araujo, A.B., Coviello, A.D., Bremner, W.J., and McKinlay, J.B. (2002). Age trends in the level of serum testosterone and other hormones in middle-aged men: longitudinal results from the Massachusetts male aging study. J. Clin. Endocrinol. Metab. *87*, 589–598.

Ferron, M., Hinoi, E., Karsenty, G., and Ducy, P. (2008). Osteocalcin differentially regulates beta cell and adipocyte gene expression and affects the development of metabolic diseases in wild-type mice. Proc. Natl. Acad. Sci. USA *105*, 5266–5270.

Ferron, M., Wei, J., Yoshizawa, T., Del Fattore, A., DePinho, R.A., Teti, A., Ducy, P., and Karsenty, G. (2010). Insulin signaling in osteoblasts integrates bone remodeling and energy metabolism. Cell *142*, 296–308.

Fukumoto, S., and Yamashita, T. (2007). FGF23 is a hormone-regulating phosphate metabolism—unique biological characteristics of FGF23. Bone *40*, 1190–1195.

Fulzele, K., Riddle, R.C., DiGirolamo, D.J., Cao, X., Wan, C., Chen, D., Faugere, M.C., Aja, S., Hussain, M.A., Brüning, J.C., and Clemens, T.L. (2010). Insulin receptor signaling in osteoblasts regulates postnatal bone acquisition and body composition. Cell *142*, 309–319.

Gray, A., Berlin, J.A., McKinlay, J.B., and Longcope, C. (1991). An examination of research design effects on the association of testosterone and male aging: results of a meta-analysis. J. Clin. Epidemiol. *44*, 671–684.

Harada, S., and Rodan, G.A. (2003). Control of osteoblast function and regulation of bone mass. Nature *423*, 349–355.

Henriksén, K., Hakovirta, H., and Parvinen, M. (1995). Testosterone inhibits and induces apoptosis in rat seminiferous tubules in a stage-specific manner: in situ quantification in squash preparations after administration of ethane dimethane sulfonate. Endocrinology *136*, 3285–3291.

Hunter, M.G., Magee-Brown, R., Dix, C.J., and Cooke, B.A. (1982). The functional activity of adult mouse Leydig cells in monolayer culture. Effect of lutropin and foetal calf serum. Mol. Cell. Endocrinol. *25*, 35–47.

Jamin, S.P., Arango, N.A., Mishina, Y., Hanks, M.C., and Behringer, R.R. (2003). Genetic studies of the AMH/MIS signaling pathway for Müllerian duct regression. Mol. Cell. Endocrinol. *211*, 15–19.

Kanazawa, I., Yamaguchi, T., Tada, Y., Yamauchi, M., Yano, S., and Sugimoto, T. (2010). Serum osteocalcin level is positively associated with insulin sensitivity and secretion in patients with type 2 diabetes. Bone, in press. Published online December 23, 2010. 10.1016/j.bone.2010.12.020.

Karsenty, G. (2006). Convergence between bone and energy homeostases: leptin regulation of bone mass. Cell Metab. *4*, 341–348.

Karsenty, G., Kronenberg, H.M., and Settembre, C. (2009). Genetic control of bone formation. Annu. Rev. Cell Dev. Biol. *25*, 629–648.

Khosla, S., Melton, L.J., III, Atkinson, E.J., and O'Fallon, W.M. (2001). Relationship of serum sex steroid levels to longitudinal changes in bone density in young versus elderly men. J. Clin. Endocrinol. Metab. *86*, 3555–3561.

Lee, N.K., Sowa, H., Hinoi, E., Ferron, M., Ahn, J.D., Confavreux, C., Dacquin, R., Mee, P.J., McKee, M.D., Jung, D.Y., et al. (2007). Endocrine regulation of energy metabolism by the skeleton. Cell *130*, 456–469.

Manolagas, S.C., Kousteni, S., and Jilka, R.L. (2002). Sex steroids and bone. Recent Prog. Horm. Res. *57*, 385–409.

Nakamura, T., Imai, Y., Matsumoto, T., Sato, S., Takeuchi, K., Igarashi, K., Harada, Y., Azuma, Y., Krust, A., Yamamoto, Y., et al. (2007). Estrogen prevents bone loss via estrogen receptor alpha and induction of Fas ligand in osteoclasts. Cell *130*, 811–823.

Nelson, L.R., and Bulun, S.E. (2001). Estrogen production and action. J. Am. Acad. Dermatol. *45*(3, Suppl), S116–S124.

Pi, M., Faber, P., Ekema, G., Jackson, P.D., Ting, A., Wang, N., Fontilla-Poole, M., Mays, R.W., Brunden, K.R., Harrington, J.J., and Quarles, L.D. (2005). Identification of a novel extracellular cation-sensing G-protein-coupled receptor. J. Biol. Chem. *280*, 40201–40209.

Pi, M., Chen, L., Huang, M.Z., Zhu, W., Ringhofer, B., Luo, J., Christenson, L., Li, B., Zhang, J., Jackson, P.D., et al. (2008). GPRC6A null mice exhibit osteopenia, feminization and metabolic syndrome. PLoS ONE *3*, e3858.

Pi, M., Parrill, A.L., and Quarles, L.D. (2010). GPRC6A mediates the non-genomic effects of steroids. J Biol. Chem. *285*, 39953–39964.

Powlin, S.S., Cook, J.C., Novak, S., and O'Connor, J.C. (1998). Ex vivo and in vitro testis and ovary explants: utility for identifying steroid biosynthesis inhibitors and comparison to a Tier I screening battery. Toxicol. Sci. *46*, 61–74.

Quigley, C.A. (2002). Editorial: The postnatal gonadotropin and sex steroid surge-insights from the androgen insensitivity syndrome. J. Clin. Endocrinol. Metab. *87*, 24–28.

Riggs, B.L., O'Fallon, W.M., Muhs, J., O'Connor, M.K., Kumar, R., and Melton, L.J., III. (1998). Long-term effects of calcium supplementation on serum parathyroid hormone level, bone turnover, and bone loss in elderly women. J. Bone Miner. Res. *13*, 168–174.

Rodan, G.A., and Martin, T.J. (2000). Therapeutic approaches to bone diseases. Science *289*, 1508–1514.

Russell, L.D., Attlin, R., Sinha Hikim, A., and Clegg, E. (1990). Histological and histopathological evaluation of the testis (Clearwater, FL: Cache River Press).

Saleem, U., Mosley, T.H., Jr., and Kullo, I.J. (2010). Serum osteocalcin is associated with measures of insulin resistance, adipokine levels, and the presence of metabolic syndrome. Arterioscler. Thromb. Vasc. Biol. *30*, 1474–1478.

Schumacher, M., Schäfer, G., Holstein, A.F., and Hilz, H. (1978). Rapid isolation of mouse Leydig cells by centrifugation in Percoll density gradients with complete retention of morphological and biochemical integrity. FEBS Lett. *91*, 333–338.

Sharpe, R.M., Maddocks, S., Millar, M., Kerr, J.B., Saunders, P.T., and McKinnell, C. (1992). Testosterone and spermatogenesis. Identification of stage-specific, androgen-regulated proteins secreted by adult rat seminiferous tubules. J. Androl. *13*, 172–184.

Sheng, Y., Tsai-Morris, C.H., Gutti, R., Maeda, Y., and Dufau, M.L. (2006). Gonadotropin-regulated testicular RNA helicase (GRTH/Ddx25) is a transport protein involved in gene-specific mRNA export and protein translation during spermatogenesis. J. Biol. Chem. *281*, 35048–35056.

Simpson, E.R. (2003). Sources of estrogen and their importance. J. Steroid Biochem. Mol. Biol. *86*, 225–230.

Simpson, E., Rubin, G., Clyne, C., Robertson, K., O'Donnell, L., Jones, M., and Davis, S. (2000). The role of local estrogen biosynthesis in males and females. Trends Endocrinol. Metab. *11*, 184–188.

Sinha Hikim, A.P., and Swerdloff, R.S. (1999). Hormonal and genetic control of germ cell apoptosis in the testis. Rev. Reprod. *4*, 38–47.

Suárez, S.S., and Osman, R.A. (1987). Initiation of hyperactivated flagellar bending in mouse sperm within the female reproductive tract. Biol. Reprod. *36*, 1191–1198.

Tsai-Morris, C.H., Koh, E., Sheng, Y., Maeda, Y., Gutti, R., Namiki, M., and Dufau, M.L. (2007). Polymorphism of the GRTH/DDX25 gene in normal and infertile Japanese men: a missense mutation associated with loss of GRTH phosphorylation. Mol. Hum. Reprod. *13*, 887–892.

Tsai-Morris, C.H., Sheng, Y., Gutti, R., Li, J., Pickel, J., and Dufau, M.L. (2010). Gonadotropin-regulated testicular RNA helicase (GRTH/DDX25) gene: cell-specific expression and transcriptional regulation by androgen in transgenic mouse testis. J. Cell. Biochem. *109*, 1142–1147.

Walker, W.H. (2009). Molecular mechanisms of testosterone action in spermatogenesis. Steroids *74*, 602–607.

Wang, G.M., O'Shaughnessy, P.J., Chubb, C., Robaire, B., and Hardy, M.P. (2003). Effects of insulin-like growth factor I on steroidogenic enzyme expression levels in mouse leydig cells. Endocrinology *144*, 5058–5064.

Wellendorph, P., and Bräuner-Osborne, H. (2004). Molecular cloning, expression, and sequence analysis of GPRC6A, a novel family C G-protein-coupled receptor. Gene *335*, 37–46.

Wellendorph, P., Hansen, K.B., Balsgaard, A., Greenwood, J.R., Egebjerg, J., and Bräuner-Osborne, H. (2005). Deorphanization of GPRC6A: a promiscuous L-alpha-amino acid receptor with preference for basic amino acids. Mol. Pharmacol. *67*, 589–597.

Yeap, B.B., Chubb, S.A., Flicker, L., McCaul, K.A., Ebeling, P.R., Beilby, J.P., and Norman, P.E. (2010). Reduced serum total osteocalcin is associated with metabolic syndrome in older men via waist circumference, hyperglycemia, and triglyceride levels. Eur. J. Endocrinol. *163*, 265–272.

Zhang, X., Odom, D.T., Koo, S.H., Conkright, M.D., Canettieri, G., Best, J., Chen, H., Jenner, R., Herbolsheimer, E., Jacobsen, E., et al. (2005). Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. Proc. Natl. Acad. Sci. USA *102*, 4459–4464.