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Oscillatory Expression of the bHLH Factor Hes1 Regulated by a Negative Feedback Loop

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Transcription of messenger RNAs (mRNAs) for Notch signaling molecules oscillates with 2-hour cycles, and this oscillation is important for coordinated somite segmentation. However, the molecular mechanism of such oscillation remains to be determined. Here, we show that serum treatment of cultured cells induces cyclic expression of both mRNA and protein of the Notch effector Hes1, a basic helix-loop-helix (bHLH) factor, with 2-hour periodicity. Cycling is cell-autonomous and depends on negative autoregulation of *hes1* transcription and ubiquitin-proteasome–mediated degradation of Hes1 protein. Because Hes1 oscillation can be seen in many cell types, this clock may regulate timing in many biological systems.

Although circadian clocks have been well characterized (*1*), other molecular clocks that regulate many biological processes, such as embryogenesis, are not known. It has been shown that mRNAs for Notch signaling molecules such as the bHLH factor Hes1 oscillate with 2-hour cycles during somite segmentation, which occurs every 2 hours (*2–9*). However, the molecular mechanism of such oscillation remains to be determined.

We have found that *hes1* transcription is induced in stationary cultured cells upon stimulation by serum (*10*). However, induced levels of expression were variable, depending on when measurements were taken. We therefore examined the time course of *hes1* mRNA induction in detail. A single serum treatment induces 2-hour cycle oscillation of *hes1* mRNA in a variety of cultured cells, such as myoblasts (C2C12) (Fig. 1A), fibroblasts (C3H10T1/2), neuroblastoma cells (PC12), and teratocarcinoma cells (F9) (*10, 11*). This oscillation continues for 6 to 12 hours, corresponding to three to six cycles (Fig. 1A).

Hes1 protein also oscillates in a 2-hour cycle after a single serum treatment (Fig. 1B). Protein oscillation is delayed by ~15 min relative to the mRNA oscillation (Fig. 1C). This time delay may reflect the time required for protein degradation. The *hes1* mRNA and Hes1 protein oscillations in cultured cells are not dependent on the inductive stimulus: They are also induced by exposure to cells expressing Delta (fig. S1), which is known to up-regulate Hes1 expression via Notch signaling (*12, 13*). *hes1* oscillation is observed in cells treated with Ara-C, an inhibitor of DNA replication, suggesting that cell cycle progression is not relevant to Hes1 oscillation (*10*).

We next examined the half-lives of *hes1* mRNA and Hes1 protein (*11*). The half-life of *hes1* mRNA was found to be 24.1 ± 1.7 min (fig. S2A) whereas that of Hes1 protein was about 22.3 ± 3.1 min (fig. S2B). The half-life of Hes1 protein is even shorter than that of c-Fos protein (~2 hours), which is known to disappear rapidly after immediate-early induction (*14*). The short half-lives for *hes1* mRNA and Hes1 protein may enable such a 2-hour cycle oscillation. The instability of *hes1* mRNA could be regulated by the 3′-untranslated region, as revealed for other *hes1*-related mRNAs (*15*).

To identify proteases responsible for Hes1 protein degradation, we tested various protease inhibitors for their ability to stabilize Hes1 protein. Application of proteasome inhibitors [lactacystin, MG132, and *N*-acetyl-Leu-Leu-norleucinal (ALLN)] (*16*) stabilized Hes1 protein and blocked serum-induced Hes1 protein oscillation, whereas other protease inhibitors [leupeptin, phenylmethylsulfonyl fluoride (PMSF), pepstatin A, and *N*-acetyl-Leu-Leu-methioninal (ALLM)] did not (Fig. 2A) (fig. S3); these findings suggest that Hes1 protein is specifically degraded by the ubiquitin-proteasome pathway. To confirm this notion, we expressed Hes1 protein with the hemagglutinin (HA) tag in C3H10T1/2 cells and analyzed it for ubiquitination (*11*). In the presence of the proteasome inhibitor lactacystin, high molecular weight bands (>100 kD) as well as a full-length Hes1 band are detected by antibody to HA (anti-HA) (Fig. 2B, lane 4). Furthermore, these high molecular weight species were found to be highly reactive to anti-ubiquitin, confirming that Hes1 protein is ubiquitinated in cells (Fig. 2B, lane 8).

We next wanted to examine the mechanism for the observed Hes1 oscillation. We previously showed that Hes1, a transcriptional repressor, negatively autoregulates its own expression by directly binding to its own promoter (*17, 18*). Thus, one likely mechanism is that serum-induced Hes1 protein represses *hes1* mRNA synthesis, which leads to rapid loss of Hes1 protein by the ubiquitin-proteasome pathway, and loss of Hes1 protein in turn relieves repression of *hes1* mRNA synthesis. In this model, the oscillation is attributable to negative autoregulation of *hes1* mRNA synthesis by Hes1 protein. If this model is correct, manipulation of the Hes1 protein level should affect *hes1* mRNA oscillation. An alternative model is that Hes1 protein is not an essential component but just an output of a primary clock. In this model, *hes1* mRNA oscillation is regulated by such a clock and not by Hes1 protein. To address this issue, we manipulated the Hes1 protein level and monitored *hes1* mRNA oscillation.

In the presence of the proteasome inhibitor MG132, *hes1* mRNA is transiently induced by a serum treatment, but it remains suppressed persistently thereafter (Fig. 3A).

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This is probably due to a constant repression of *hes1* transcription by persistently high Hes1 protein levels. To directly show that Hes1 protein represses *hes1* mRNA synthesis, we constitutively expressed Hes1 protein by introducing the expression vector carrying only the Hes1 coding region, and we monitored the endogenous *hes1* mRNA with the probe for *hes1* noncoding region. When Hes1 protein is constitutively expressed from the expression vector, the endogenous *hes1* mRNA is kept at the minimum level and does not respond to serum treatment (Fig. 3B). Thus, sustained increase of Hes1 protein represses *hes1* mRNA synthesis, reflecting negative autoregulation. These results indicate that degradation of Hes1 protein is required for *hes1* mRNA increase.

We next examined whether inhibition of de novo protein synthesis affects *hes1* mRNA oscillation. Treatment with cycloheximide, an inhibitor of translation, leads to sustained increase of *hes1* mRNA and blocks its oscillation (Fig. 3C). Thus, de novo protein synthesis is required for *hes1* mRNA repression. However, because cycloheximide frequently stabilizes mRNAs, the observed block of oscillation could be simply due to stabilization of *hes1* mRNA. To determine whether Hes1 protein activity is required for *hes1* mRNA repression, we next introduced the expression vector carrying only the coding region of a dominant-negative form of Hes1 (dnHes1), which was previously shown to suppress Hes1 protein activity by forming a non–DNA-binding heterodimer complex (*19*), and monitored the endogenous *hes1* mRNA with the probe for *hes1* noncoding region. Overexpression of dnHes1 also leads to sustained increase of *hes1* mRNA and blocks serum-induced *hes1* mRNA oscillation (Fig. 3D). Thus, Hes1 protein activity is required for reduction of *hes1* mRNA level. These results together demonstrated that *hes1* mRNA oscillation requires both de novo synthesis and degradation of Hes1 protein, supporting the hypothesis that Hes1 is an essential component of a 2-hour cycle clock.

We next asked whether the same mechanism applies to *hes1* mRNA oscillation in the presomitic mesoderm (PSM). As in cultured cells, treatment with MG132 down-regulates

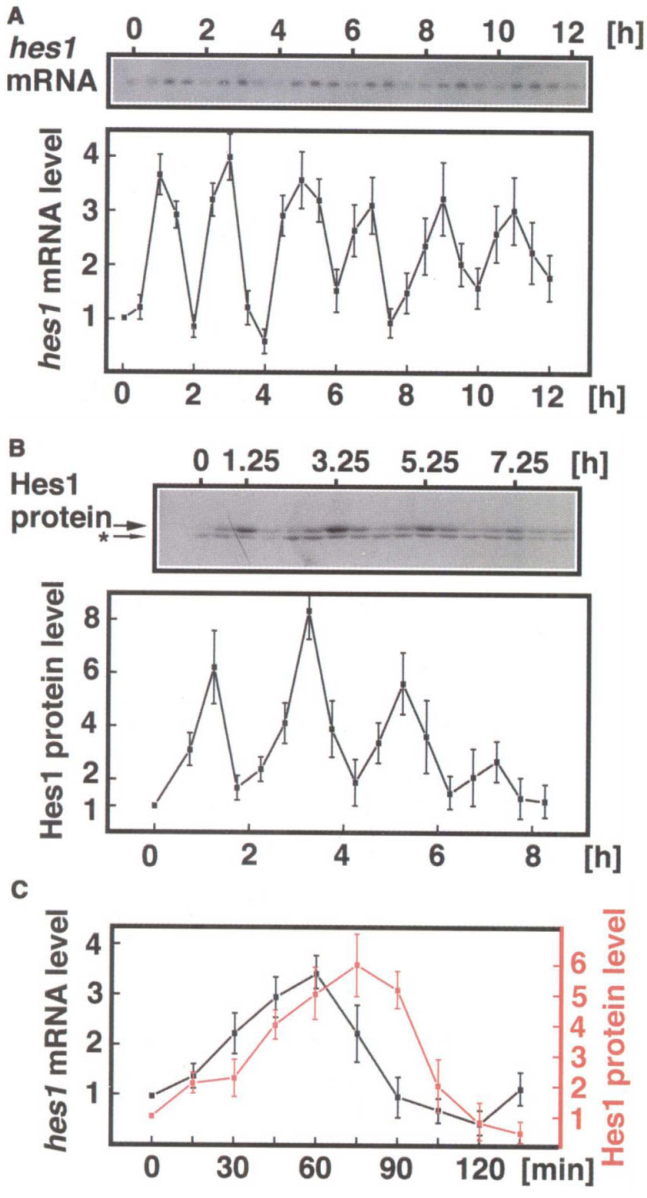

Fig. 1. Oscillation of *hes1* mRNA and Hes1 protein in cultured cells. (**A**) After serum treatment (t = 0), *hes1* mRNA level was examined every 30 min. *hes1* mRNA exhibits a 2-hour cycle oscillation. (**B**) After serum treatment, Hes1 protein level was examined at t = 45 min (0.75 hour) and every 30 min afterward. Hes1 protein also exhibits a 2-hour cycle oscillation; a nonspecific band (*) is relatively constant. (**C**) Comparison of the time course of *hes1* mRNA (black line) and Hes1 protein oscillation (red line). Error bars indicate SE for each data point.

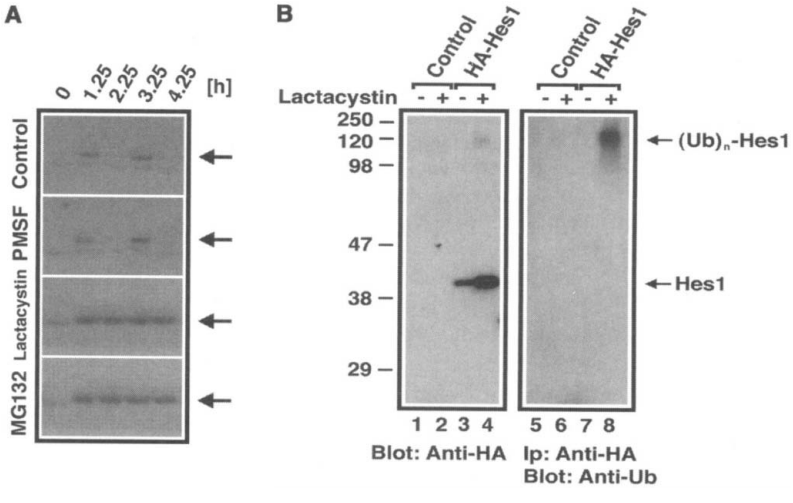

Fig. 2. Degradation of Hes1 protein by the ubiquitin-proteasome system. (**A**) Serum-induced Hes1 protein oscillation is blocked by proteasome inhibitors [lactacystin (100 μM), MG132 (100 μM)], but not by other protease inhibitors such as PMSF (1 mM). (**B**) C3H10T1/2 cells were transfected with pEF-BOS carrying no insert (lanes 1, 2, 5, and 6) or HA-Hes1 cDNA (lanes 3, 4, 7, and 8) and cultured overnight in the presence (+) or absence (–) of lactacystin (20 μM). In lanes 1 to 4, whole-cell extracts were probed with anti-HA. In the presence of lactacystin (20 μM), Hes1 protein is stabilized and higher molecular weight bands also appear (lane 4). In lanes 5 to 8, whole-cell extracts were immunoprecipitated with anti-HA and probed with anti-ubiquitin. High molecular weight species are highly reactive to anti-ubiquitin.

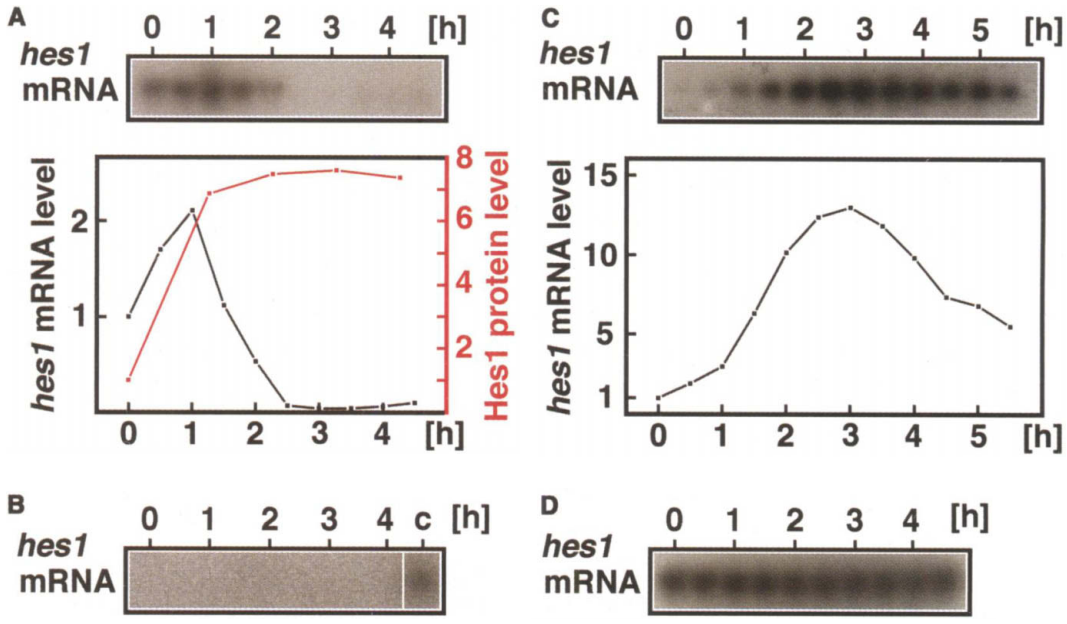

Fig. 3. *hes1* mRNA oscillation is affected by Hes1 protein levels. (**A**) In the presence of MG132 (100 μM), *hes1* mRNA (black line) is increased after serum treatment but then kept down-regulated after Hes1 protein is stabilized (red line, from Fig. 2A). (**B**) C3H10T1/2 cells were transiently transfected with the expression vector carrying Hes1 coding region alone on the previous day. The endogenous *hes1* mRNA does not increase after serum treatment. Lane c indicates transfection of the expression vector carrying no insert. (**C**) C3H10T1/2 cells were cultured in the presence of cycloheximide (10 μM). Serum treatment leads to sustained increase of *hes1* mRNA. (**D**) C3H10T1/2 cells were transfected with the expression vector of dnHes1 on the previous day. dnHes1 leads to sustained increase of the endogenous *hes1* mRNA and blocks serum-induced *hes1* mRNA oscillation.

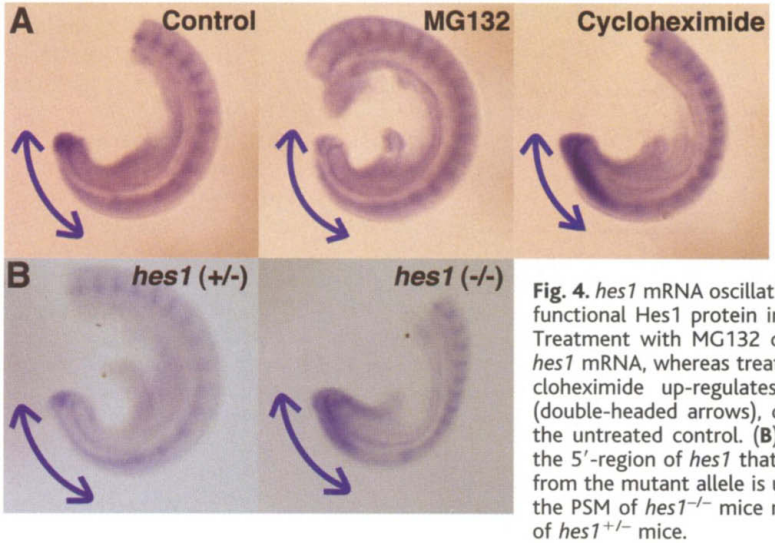

Fig. 4. *hes1* mRNA oscillation depends on functional Hes1 protein in the PSM. (**A**) Treatment with MG132 down-regulates *hes1* mRNA, whereas treatment with cycloheximide up-regulates *hes1* mRNA (double-headed arrows), compared with the untreated control. (**B**) Expression of the 5′-region of *hes1* that is not deleted from the mutant allele is up-regulated in the PSM of $hes1^{-/-}$ mice relative to that of $hes1^{+/-}$ mice.

hes1 mRNA, whereas treatment with cycloheximide up-regulates *hes1* mRNA (Fig. 4A), suggesting that de novo protein synthesis and degradation are required for cyclic expression of *hes1* mRNA in the PSM. Furthermore, expression of the 5′-region of *hes1* gene, which is not deleted from the mutant allele and expressed from the *hes1* promoter, is up-regulated in the PSM of $hes1^{-/-}$ mice relative to that of $hes1^{+/-}$ mice (Fig. 4B). Thus, in the absence of functional Hes1 protein, expression from the *hes1* promoter is up-regulated and thereby *hes1* mRNA oscillation is blocked; this finding indicates that the same oscillation mechanism works in cultured cells and the PSM.

An oscillation with a cycle of a few hours was previously generated by the artificial network consisting of three transcriptional repressors in *E. coli*, and this oscillation is depicted by a mathematical model (*20*). For Hes1 oscillation, a simple negative feedback loop, in which Hes1 represses transcription from the *hes1* promoter, would be insufficient to maintain a stable oscillation, because this system would rapidly fall into equilibrium. However, by postulating a Hes1-interacting factor, a Hes1 oscillator can be readily simulated by a set of three simple differential equations (fig. S4). According to the equations, alteration of the synthesis and degradation rates should change the period of oscillation. In agreement with this prediction, a temperature shift from 37°C to 30°C, which lowers both the synthesis and degradation rates, prolongs the period of *hes1* mRNA oscillation (*10*).

In the mouse PSM, expression of not only *hes1*-related genes but also other Notch signaling molecules such as *lunatic fringe* (*lfng*) oscillates (*4–9, 21–25*), raising the possibility that the genetic loop (Lfng → Hes1 → Lfng) constitutes the oscillation. However, *lfng* is not expressed in the cultured cells that we used, and misexpression of *lfng* does not affect *hes1* mRNA oscillation in these cells (*10*), indicating that serum-induced Hes1 oscillation does not depend on *lfng* oscillation. Interestingly, in the PSM, *lfng* oscillation depends on cyclic promoter activation and repression (*26, 27*) and is regulated by negative feedback (*28*), indicating that both Hes1 and Lfng oscillations are controlled by a similar mechanism. Because Hes1 oscillation occurs in many cell types, this clock—which was originally identified in the PSM—is widely distributed and could regulate timing in many biological systems. It has been shown that serum treatment induces circadian oscillation in cultured cells (*29*). Thus, many cell types appear to carry at least two molecular oscillators.

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Figs. S1 to S4

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Reversal of Bone Loss in Mice by Nongenotropic Signaling of Sex Steroids

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We show that sex steroids protect the adult murine skeleton through a mechanism that is distinct from that used to preserve the mass and function of reproductive organs. The classical genotropic actions of sex steroid receptors are dispensable for their bone protective effects, but essential for their effects on reproductive tissues. A synthetic ligand (4-estren-3α,17β-diol) that reproduces the nongenotropic effects of sex steroids, without affecting classical transcription, increases bone mass and strength in ovariectomized females above the level of the estrogen-replete state and is at least as effective as dihydrotestosterone in orchidectomized males, without affecting reproductive organs. Such ligands merit investigation as potential therapeutic alternatives to hormone replacement for osteoporosis of bone mass in both women and men.

Estrogens and androgens exert many biological effects that cannot be explained by interactions of their receptors with DNA (*1*, *2*). Heretofore, there has been no evidence that such "nongenotropic" actions of sex steroids (*3*, *4*) are of biological relevance in vivo. We have recently elucidated potent anti-apoptotic effects of estrogens and androgens on murine osteoblasts and osteocytes (*5*). These effects are due to stimulation of the Src/Shc/ERK and repression of the JNK signaling cascades via a nongenotropic action of the classical sex steroid receptors, leading to downstream modulation of the activity of transcription factors such as Elk-1, C/EBPβ (NF-IL6), CREB, and c-Fos/c-Jun (*6*). Unlike classical effects of sex steroids on reproductive tissues, all these actions are non–sex-specific, require only the ligand-binding domain of the receptor, and are eliminated by nuclear targeting. Moreover, these nongenotropic actions can be faithfully reproduced by 4-estren-3α,17β-diol, a synthetic compound referred to hereafter as estren, which has no classical transcriptional activity. Conversely, 1,2,5-tris(4-hydroxylphenyl)-4-propylpyrazole, referred to hereafter as pyrazole, has potent transcriptional activity but minimal (if any) effects on ERK or JNK kinases.

We compared the effects of estren to those of 17β-estradiol (E_2) or dihydrotestosterone (DHT) on bone and reproductive tissues of adult Swiss Webster mice (*7*). E_2, DHT, and estren, but not pyrazole, not only attenuated osteoblast apoptosis but also stimulated osteoclast apoptosis, with identical potency in primary cultures of cells from female or male mice, which is consistent with a non–sex-specific mechanism of action (Fig. 1A). Furthermore, irrespective of the sex of the mouse, a replacement dose of either E_2 or DHT, or administration of estren to gonadectomized females or males, prevented gonadectomy-induced osteoblast apoptosis in the lumbar vertebrae (Fig. 1B).

For the in vivo studies, we used a ~300-fold higher dose of estren, as compared to E_2, on the basis of its lower binding affinity for the estrogen receptor (ER) (fig. S1). In spite of this, estren, unlike E_2, did not stimulate estrogen response element–mediated transcription of the C3 gene in the uterus, in agreement with its in vitro properties (*5*) (Fig. 1C). None of the compounds affected body weight. Estren was at least as effective as estradiol in preserving global and spinal bone mineral density (BMD) in females (Fig. 1D and fig. S2). In addition, ovariectomized (OVX) mice receiving estren exhibited greater BMD change in the hindlimb, not only compared with the OVX mice receiving E_2 replacement but also compared with the estrogen-replete sham controls, suggesting an anabolic effect (that is, the addition of new bone) at this site of predominantly cortical bone. Estren also appeared to be at least as effective as DHT replacement in orchidecto-

Table 1. Increased trabecular and cortical width, osteoblast number, and serum osteocalcin after treatment with estren. Histomorphometric analysis of L1 to L4 vertebrae from 6-month-old females and serum osteocalcin levels from the 6- and 8-month-old females are shown. Data are means ± SD.

Parameter	Sham	OVX	OVX + E_2	OVX + estren
Cortical width (μm)	117 ± 13	107 ± 10	108 ± 10	138 ± 28*‡
Bone area per tissue area (%)	23 ± 6	18 ± 3	15 ± 3†	20 ± 5
Trabecular width (μm)	59 ± 7*	48 ± 4	41 ± 6†	56 ± 7‡
Osteoid perimeter per bone perimeter (%)	19.3 ± 5.3*	25.3 ± 5.1	4.3 ± 2.9*†	12.3 ± 2*†‡
Osteoblast number per bone perimeter (*N*/mm)	14.4 ± 4	20.3 ± 6.9	3.6 ± 2.8*†	10.9 ± 3*‡
Osteoclast number per bone perimeter (*N*/mm)	2.3 ± 0.4	3.4 ± 1.6	0.9 ± 0.3*†	1.8 ± 0.6*
Bone formation rate per bone area (%/day)	0.457 ± 0.262	0.915 ± 0.475	0.080 ± 0.048*	0.158 ± 0.108*
Serum osteocalcin (ng/ml)	126 ± 24*	150 ± 31	81 ± 14*	157 ± 19*†‡

*$P < 0.05$ versus OVX. †$P < 0.05$ versus sham. ‡$P < 0.05$ versus OVX + E_2.

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