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SHORT COMMUNICATION

Adrenocorticotropic hormone at pathophysiological concentration modulates the proliferation and differentiation of bone cells

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Summary *Background/purpose:* Adrenocorticotropic hormone (ACTH) plays a vital role in maintaining the function of the hypothalamic–pituitary–adrenal axis. Recent studies have demonstrated that ACTH directly affects the proliferation and differentiation of bone cells. However, the ACTH concentrations used in these studies appear to be markedly higher than the physiological concentrations. Here, we investigated whether ACTH at pathophysiological concentration affects the proliferation and differentiation of osteoblasts and osteoclasts.
Materials and methods: We evaluated the effect of ACTH at pathophysiological concentration on osteoclasts using tartrate-resistant acid phosphatase staining and on osteoblasts using alkaline phosphatase activity assay. Additionally, we conducted reverse transcriptase-polymerase chain reaction analysis.
Results: We found that at pathophysiological concentration, ACTH does not affect osteoblast proliferation and inhibits osteoblast differentiation. Moreover, we showed that at pathophysiological concentration, ACTH does not affect the proliferation of bone marrow macrophages, but promotes differentiation of osteoclasts and induces expression of genes involved in bone resorption.

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Conclusion: Taken together, our findings suggest that ACTH modulates the proliferation and differentiation of bone cells *in vitro* at pathophysiological concentration.

Introduction

Tooth extraction is a common treatment modality for dental problems and is accompanied by damage to the bone tissue. In addition, dentists are increasingly examining patients with depressive disorder, which is the fourth leading cause of disability worldwide and is expected to be the second leading cause by 2020.[1] As a defense response against stress factors that lead to diseases such as depressive disorder, including conditions of mental stress, the hypothalamo—pituitary—adrenal (HPA) axis is activated to maintain homeostasis.[2] Therefore, there is a need to determine the influence of stress hormones on bone metabolism and how such mental stress affects the healing of bone tissue in patients with depressive disorder who undergo tooth extraction.

Bone tissue is continuously regulated to maintain homeostasis via endocrine systems, the nervous system, and mechanical adaptation.[3–5] Bone homeostasis refers to the balance between bone tissue formation by osteoblasts and its degradation by osteoclasts. The function of these cells is regulated by several factors, including cytokines and hormones. Accumulating evidence has demonstrated that key players in bone homeostasis include pituitary hormones such as growth hormone, follicle-stimulating hormone, thyroid-stimulating hormone, prolactin, and oxytocin.[6] Adrenocorticotropic hormone (ACTH), which stimulates the secretion of glucocorticoid from the adrenal cortex, is one such stress hormone regulating the HPA axis. Hypersecretion of ACTH has been observed in patients suffering from major depressive disorder,[7] suggesting that the effects of high concentrations of ACTH on bone are worthy of investigation.

Recent studies demonstrated that ACTH affects the proliferation and differentiation of bone cells. ACTH at 10^{-9}M promotes proliferation of human primary osteoblasts.[8] ACTH at 10^{-8}M promotes collagen type 1 expression in human osteosarcoma cell line[9] and induces expression of osteoblastic differentiation-associated genes in murine primary stromal cells.[10] ACTH at 10^{-8}M has also been reported to promote osteoclast differentiation in murine osteoclasts.[10] According to a previous study, however, the concentration of ACTH was 281 pg/mL (6.2×10^{-11}M) in wild-type mice and 1394 pg/mL (3.1×10^{-10}M) in melanocortin 2 receptor (MC2R; an ACTH receptor)-knockout mice, which are animal models for familial glucocorticoid deficiency.[11] The former concentration of ACTH is considered the "physiological" concentration, while the latter is considered the "pathophysiological" concentration. In MC2R-knockout mice, the ACTH level increased because of deficiency in the negative feedback provided by corticosterone, suggesting that this level is considered the maximum level of ACTH possible. However, the ACTH concentration of 10^{-8}M used in previous studies is 100-fold higher than the level found in MC2R-knockout mice. Furthermore, other studies showed that the ACTH level was 6.7 pg/mL (1.5×10^{-12}M) in mice exposed to a novel environmental stress[12] and 100 pg/mL (2.2×10^{-11}M) in corticotropin-releasing factor transgenic mice.[13]

In this study, we investigated whether ACTH affects the proliferation and differentiation of osteoblasts and osteoclasts at pathophysiological concentration.

Materials and methods

Reagents

Recombinant human ACTH was purchased from Abcam (Cambridge, UK).

Animals

All mice (C57BL/6) were obtained from Tokyo Laboratory Animals Science, fed normal laboratory chow, and reared in an environmentally controlled clean room. Experiments were conducted according to the institutional guidelines for ethical animal experiments.

Cell culture

Mouse osteoclasts were generated using previously established *in vitro* protocols.[14] In short, bone marrow cells obtained from the tibia of 8- to 10-week-old female mice were cultured in α-minimal essential medium (Wako; Tokyo, Japan) containing 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO, USA) for 16—20 hours, and nonadherent cells were harvested and cultured at a concentration of 1×10^5 cells/cm^2 for 2 days with 10 ng/mL macrophage colony-stimulating factor (M-CSF) (Peprotech, Rocky Hill, NJ, USA). Adherent cells were used as bone marrow macrophages (BMMs). To obtain osteoclasts, BMMs were further cultured with 100 ng/mL receptor activator of nuclear factor kappa-B ligand (RANKL) (Oriental Yeast, Tokyo, Japan) and 10 ng/mL M-CSF. We used M-CSF and RANKL at these concentrations throughout the study. Murine primary osteoblasts were isolated from the calvaria collected from newborn mice as previously described.[15] Neonatal mouse calvaria were dissected free of adherent soft tissue, washed in phosphate-buffered saline, and sequentially digested with 0.2% dispase and 0.1% collagenase. We used the murine preosteoblastic cell line MC3T3-E1. Both primary osteoblasts and MC3T3-E1 cells were maintained in α-minimal essential medium containing 10%

fetal bovine serum. For the osteoblastic differentiation assay, cells were cultured in a conditioned medium containing 50 μg/mL ascorbic acid and 10 mM β-glycerophosphate in a growth medium. The medium was replaced with fresh medium every 2–3 days. All cultures were maintained at 37°C in humidified air containing 5% CO_2.

Tartrate-resistant acid phosphatase staining

Recombinant ACTH was added at the same time as RANKL during the procedure described above. BMMs cultured with M-CSF and RANKL for 5 days were fixed with 10% formalin for 5 minutes to stain for tartrate-resistant acid phosphatase (TRAP). Thereafter, they were refixed with ethanol:acetone (50:50 v/v) for 1 minute and incubated in acetate buffer (pH 4.8) containing naphthol AS-MX phosphate (Sigma-Aldrich), fast red violet LB salt (Sigma-Aldrich), and 50mM sodium tartrate at room temperature. TRAP-positive, multinucleated cells with more than three nuclei were counted as osteoclasts. Results are representative of more than four individual experiments.

Measurement of alkaline phosphatase activity

Cells, at a density of 2×10^4 cells, were placed in 24-well plates. After reaching confluence, cells were incubated in a conditioned medium with ACTH at the indicated concentration for 7 days. Alkaline phosphatase (ALP) activity was assayed (Wako) as previously described.[15] The measurements are expressed as the means of three independent experiments, with each data point based on four replicates.

Reverse transcriptase-polymerase chain reaction

Total RNA was extracted from cells using ISOGEN (Nippon Gene, Tokyo, Japan) and was subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) using a SuperScript One-Step RT-PCR kit (Thermo Scientific, Wilmington, DE, USA) as previously described.[14] The gene-specific primer pairs used were matrix metalloproteinase 9 (*MMP-9*) sense, 5′-GGCCTGGAAGATGATGTTGT-3′, and *MMP-9* antisense, 5′-AATGGTGAGGTTTTGCGTTC-3′; and *β-actin* sense, 5′-AGAAGGACTCCTATGTGGGTGA-3′, and *β-actin* antisense, 5′-CATGATCTGGGTCATCTTTTCA-3′. In this study, *β-actin* was used as a loading control. All samples were examined in triplicate assays.

Quantitative real-time RT-PCR

To validate changes in gene expression, quantitative real-time RT-PCR analysis was performed using the Applied Biosystems Prism 7900HT Sequence Detection System (Thermo Scientific) according to the manufacturer's instructions, as previously described.[14] Total RNA was extracted from cells using ISOGEN (Nippon Gene). We used a TaqMan-based detection system. The reverse-transcriptase reaction was performed using High Capacity RNA-to-cDNA Master Mix (Thermo Scientific). We performed PCR amplification with real-time detection using TaqMan Gene Expression Master Mix (Thermo Scientific) and TaqMan Gene Expression Assays (Thermo Scientific) for *Bglap* (osteocalcin gene) (Mm03413826_mH) and *GAPDH* (Mm03302249_g1), which was used as an endogenous control. Values were normalized to *GAPDH* using the $2^{-\Delta\Delta Ct}$ method. All samples were examined in triplicate assays.

Statistical analysis

Comparisons between two groups were analyzed using Student *t* tests, and comparisons among three groups were analyzed using one-way analysis of variance and Bonferroni–Dunn methods ($P < 0.05$). All values are represented as mean ± the standard error of the mean. Results are representative examples of more than three independent sets of experiments.

Results

Pathophysiological concentration of ACTH did not affect proliferation but inhibited osteoblast differentiation

We assessed whether ACTH at 3×10^{-10}M induced proliferation and ALP activity in osteoblasts. Surprisingly, this concentration significantly inhibited ALP activity and did not promote proliferation of osteoblasts (Figs. 1A and 1B). We also found that expression of *Bglap*, an osteoblast differentiation marker, was significantly downregulated at this ACTH concentration (Fig. 1C).

ACTH at pathophysiological concentration did not affect proliferation of BMMs but promoted differentiation of osteoclasts

We examined whether ACTH at 3×10^{-10}M would also induce osteoclastogenesis. As expected, TRAP-positive cell formation was significantly increased by ACTH at 3×10^{-10}M (Fig. 2A). Next, we examined whether ACTH at 3×10^{-10}M induced expression of *MMP-9*, which encodes a marker for bone resorption. After incubation with M-CSF and RANKL for 5 days, RT-PCR analysis revealed that *MMP-9* expression was more strongly induced by ACTH at 3×10^{-10}M than by the control (Fig. 2B). Finally, we assessed whether ACTH at 3×10^{-10}M affected proliferation of BMMs, and found that ACTH did not affect cell proliferation at this concentration (Fig. 2C).

Discussion

ACTH is an agonist for five melanocortin receptors (known as MC1R through MC5R), all of which are present in bone cells.[8] Although ACTH is capable of binding to these receptors,[16] only MC2R is identified as a functional receptor in osteoblast differentiation.[10] ACTH induces vascular endothelial growth factor expression in osteoblasts via MC2R to promote osteoblast differentiation. However, ACTH at both 1×10^{-9}M and 1×10^{-10}M concentrations tends to inhibit collagen 1 expression in human osteoblastic-like cell line.[9] Our results concur with this observation, where ACTH at 3×10^{-10}M inhibited ALP activity and osteocalcin

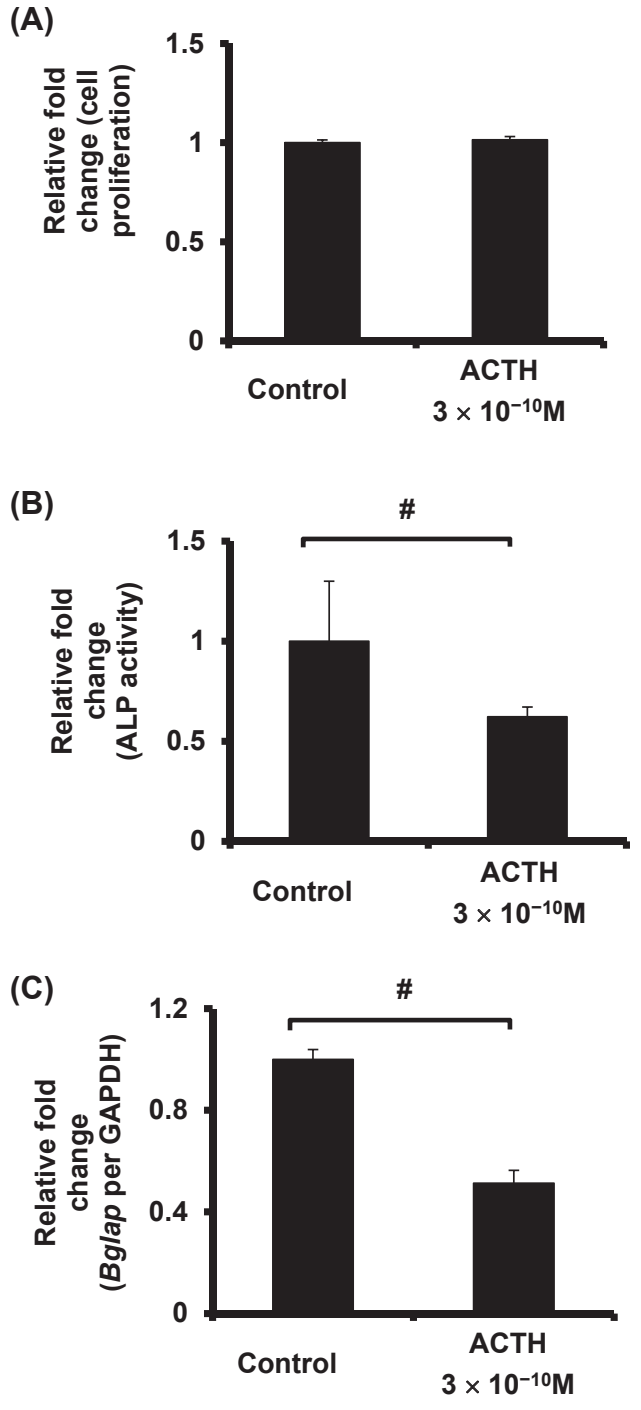

Figure 1 Inhibition of osteoblast differentiation by ACTH at pathophysiological concentration. (A) Effect of ACTH (3×10^{-10}M) on proliferation of murine primary osteoblasts incubated for 3 days. (B) Effect of ACTH (3×10^{-10}M) on ALP activity in murine primary osteoblasts incubated for 7 days. (C) Quantitative RT-PCR analysis of *Bglap* expression in MC3T3-E1 cells incubated for 14 days. Data are expressed as mean ± SEM. The results shown are representative of four independent experiments. $^{\#}$ $P < 0.05$. ACTH = adrenocorticotropic hormone; ALP = alkaline phosphatase; RT-PCR = reverse transcriptase-polymerase chain reaction; SEM = standard error of the means; GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

expression in osteoblasts (Fig. 1). On the other hand, ACTH at 1×10^{-8}M promoted osteoclast differentiation.[10] In our study, ACTH at 3×10^{-10}M increased the formation of TRAP-positive cells (Fig. 2).

We showed that, at pathophysiological concentration, ACTH inhibits osteoblast differentiation and promotes osteoclast differentiation, suggesting that ACTH negatively regulates bone cell function *in vitro*. On the other hand, Zaidi et al[10] demonstrated that exogenous ACTH (at physiological concentration) prevents glucocorticoid-induced osteonecrosis *in vivo*, and a high concentration (1×10^{-8}M) of ACTH (higher than pathophysiological concentration) promotes osteoblast differentiation *in vitro*. Their results may support the notion that ACTH positively regulates bone metabolism *in vivo*. They considered that ACTH administration promotes osteoblast differentiation at local sites. This raises the question whether or not local ACTH concentration reaches a level higher than physiological concentration (1×10^{-8}M) due to the administration of ACTH at physiological concentration. We speculate that systemic action of ACTH may exert anabolic effects on bone metabolism via an as-yet-unknown mechanism, including via other melanocortin receptors that ACTH is capable of binding to.[16]

ACTH is encoded by the pro-opiomelanocortin (*POMC*) gene, which encodes numerous peptide hormones, including α-, β-, and γ-melanocyte-stimulating hormone, β-lipotropin, and β-endorphin. Some hormones directly affect bone metabolism. For example, systemic administration of α-melanocyte-stimulating hormone increases the bone turnover rate and decreases bone volume.[17] In a previous study with POMC-knockout mice, cortical thickness was significantly increased, while changes in trabecular bone were not significant.[18] Moreover, patients with familial glucocorticoid deficiency, a rare autosomal-recessive disease characterized by glucocorticoid deficiency, exhibit tall stature and have advanced bone maturation in the radius and phalanges, with a delay seen in the carpal bones.[19] This suggests that MC2R signaling is associated with bone homeostasis. These studies indicate that the POMC family of proteins and MC2R play critical roles in bone metabolism.

Excess levels of glucocorticoid result in inhibition of osteoblast differentiation and promotion of osteoclast differentiation.[20] High concentrations of glucocorticoid and ACTH in serum have been observed in patients with diseases such as Cushing syndrome and major depressive disorder.[21,22] It is well known that Cushing syndrome leads to the development of osteoporosis.[21] A recent study has shown a strong association between major depressive disorder and low bone mass in adults.[23] Traumatic stress, which activates the HPA axis, has also been shown to exert a negative impact on mouse bone development *in vivo*.[24] These studies strongly suggest that stress hormones negatively affect the function of bone cells, and that the HPA axis plays a pivotal role in the regulation of bone metabolism. Our study may provide important knowledge about the relationship between stress hormones and bone metabolism. On the other hand, our study has a limitation because the results are obtained from *in vitro* experiments.

From the viewpoint of dental medicine, it is noteworthy to study the delay in healing of bone tissue after surgical

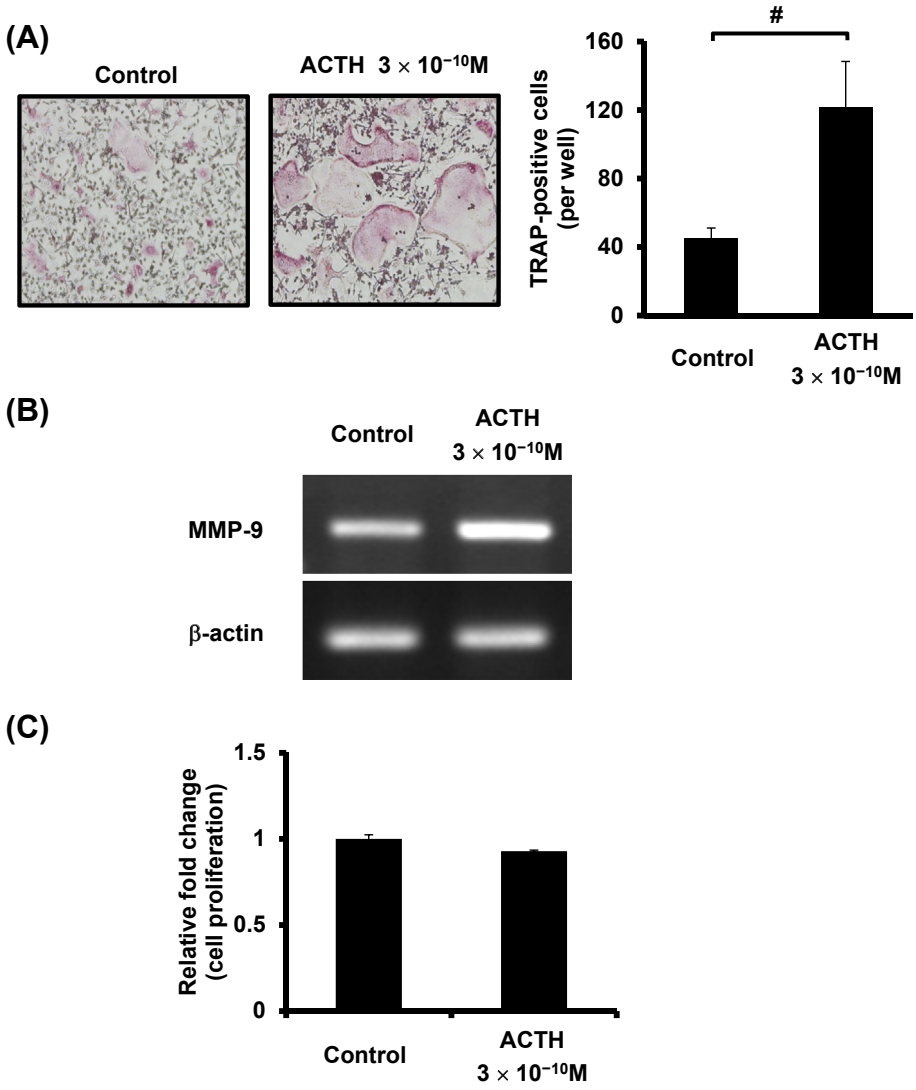

Figure 2 Promotion of osteoclast differentiation by ACTH at pathophysiological concentration. (A) Effect of ACTH (3×10^{-10}M) on differentiation of osteoclasts incubated for 5 days: TRAP staining (left panel) and the number of TRAP-positive multinucleated cells formed (right panel). (B) Effect of ACTH (3×10^{-10}M) on *MMP-9* expression in osteoclasts incubated for 5 days using RT-PCR analysis. Here, *β-actin* was used as a loading control. (C) Effect of ACTH (3×10^{-10}M) on proliferation of BMMs incubated for 3 days. Data are expressed as means ± SEM. The results shown are representative of four independent experiments. $^{\#}$ $P < 0.05$. ACTH = adrenocorticotropic hormone; MMP-9 = matrix metalloproteinase 9; RT-PCR = reverse transcriptase-polymerase chain reaction; SEM = standard error of the means; TRAP = tartrate-resistant acid phosphatase.

dental treatments, such as tooth extraction, in patients with major depressive disorder. Further studies are needed to clarify how ACTH regulates bone metabolism using genetically engineered animals such as MC2R-knockout mice.

ACTH at pathophysiological concentration does not affect osteoblast proliferation but inhibits osteoblast differentiation. Moreover, it does not affect proliferation of BMMs, but promotes differentiation of osteoclasts and induces upregulation of bone resorption-related gene.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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References

1. Kessler RC, Bromet EJ. The epidemiology of depression across cultures. *Annu Rev Public Health* 2013;34:119–38.
2. Faravelli C, Lo Sauro C, Lelli L, et al. The role of life events and HPA axis in anxiety disorders: a review. *Curr Pharm Des* 2012; 18:5663–74.
3. Pi M, Quarles LD. Novel bone endocrine networks integrating mineral and energy metabolism. *Curr Osteoporos Rep* 2013;11: 391–9.

4. Sharan K, Yadav VK. Hypothalamic control of bone metabolism. *Best Pract Res Clin Endocrinol Metab* 2014;28:713—23.
5. Meakin LB, Price JS, Lanyon LE. The contribution of experimental *in vivo* models to understanding the mechanisms of adaptation to mechanical loading in bone. *Front Endocrinol (Lausanne)* 2014;5:154.
6. Colaianni G, Cuscito C, Colucci S. FSH and TSH in the regulation of bone mass: the pituitary/immune/bone axis. *Clin Dev Immunol* 2013;2013:382698.
7. Linkowski P, Mendlewicz J, Kerkhofs M, et al. 24-hour profiles of adrenocorticotropin, cortisol, and growth hormone in major depressive illness: effect of antidepressant treatment. *J Clin Endocrinol Metab* 1987;65:141—52.
8. Zhong Q, Sridhar S, Ruan L, et al. Multiple melanocortin receptors are expressed in bone cells. *Bone* 2005;36:820—31.
9. Isales CM, Zaidi M, Blair HC. ACTH is a novel regulator of bone mass. *Ann N Y Acad Sci* 2010;1192:110—6.
10. Zaidi M, Sun L, Robinson LJ, et al. ACTH protects against glucocorticoid-induced osteonecrosis of bone. *Proc Natl Acad Sci U S A* 2010;107:8782—7.
11. Chida D, Nakagawa S, Nagai S, et al. Melanocortin 2 receptor is required for adrenal gland development, steroidogenesis, and neonatal gluconeogenesis. *Proc Natl Acad Sci U S A* 2007;104:18205—10.
12. Karanth S, Linthorst AC, Stalla GK, Barden N, Holsboer F, Reul JM. Hypothalamic—pituitary—adrenocortical axis changes in a transgenic mouse with impaired glucocorticoid receptor function. *Endocrinology* 1997;138:3476—85.
13. Stenzel-Poore MP, Cameron VA, Vaughan J, Sawchenko PE, Vale W. Development of Cushing's syndrome in corticotropin-releasing factor transgenic mice. *Endocrinology* 1992;130:3378—86.
14. Enoki Y, Sato T, Tanaka S, et al. Netrin-4 derived from murine vascular endothelial cells inhibits osteoclast differentiation *in vitro* and prevents bone loss *in vivo*. *FEBS Lett* 2014;588:2262—9.
15. Sato T, Abe T, Chida D, et al. Functional role of acetylcholine and the expression of cholinergic receptors and components in osteoblasts. *FEBS Lett* 2010;584:817—24.
16. Clark AJ, Weber A. Adrenocorticotropin insensitivity syndromes. *Endocr Rev* 1998;19:828—43.
17. Cornish J, Callon KE, Mountjoy KG, et al. Alpha-melanocyte-stimulating hormone is a novel regulator of bone. *Am J Physiol Endocrinol Metab* 2003;284:E1181—90.
18. Costa JL, Watson M, Callon KE, Hochgeschwender U, Cornish J. Systemic and local regulation of skeletal metabolism. Analysis of bone in POMC knockout mice. *Bone* 2010;46:1465—6.
19. Clark AJ, Metherell LA. Mechanisms of disease: the adrenocorticotropin receptor and disease. *Nat Clin Pract Endocrinol Metab* 2006;2:282—90.
20. Henneicke H, Gasparini SJ, Brennan-Speranza TC, Zhou H, Seibel MJ. Glucocorticoids and bone: local effects and systemic implications. *Trends Endocrinol Metab* 2014;25:197—211.
21. Toth M, Grossman A. Glucocorticoid-induced osteoporosis: lessons from Cushing's syndrome. *Clin Endocrinol (Oxf)* 2013;79:1—11.
22. Glynn LM, Davis EP, Sandman CA. New insights into the role of perinatal HPA-axis dysregulation in postpartum depression. *Neuropeptides* 2013;47:363—70.
23. Calarge CA, Butcher BD, Burns TL, Coryell WH, Schlechte JA, Zemel BS. Major depressive disorder and bone mass in adolescents and young adults. *J Bone Miner Res* 2014;29:2230—7.
24. Yu H, Watt H, Kesavan C, Mohan S. The negative impact of single prolonged stress (SPS) on bone development in mice. *Stress* 2013;16:564—70.