Endocrine, *vol. 17, no. 1, 37–42, February 2002* 0969–711X/02/17:37–42/$11.50

Genetic Approaches to Determine the Role of Glucocorticoid Signaling in Osteoblasts

John R. Harrison,[1] Henning W. Woitge,[2] and Barbara E. Kream[2]

Departments of [1]Orthodontics, School of Dental Medicine, and [2]School of Medicine, University of Connecticut Health Center, Farmington, CT

A variety of in vivo and in vitro experimental models have been used to explore the effects of glucococorticoids in bone. Chronically high levels of glucocorticoids typically decrease bone mass in humans and animals and inhibit markers of bone formation in organ and cell cultures. However, under certain experimental conditions, glucocorticoids can stimulate osteoblast differentiation and bone formation in vitro. The relevance of these effects seen in culture models to the role of endogenous glucocorticoids in bone remains unclear. In this article, we briefly review possible pathways for the opposing effects of glucocorticoids on bone formation and propose several genetic loss-of-function mouse models in which disruption of glucocorticoid signaling in cells of the osteoblast lineage would provide a means to determine the role of endogenous glucocorticoids in bone.

Key Words: Glucocorticoids; bone; osteoblast; Cre/loxP recombination; 11β-hydroxysteroid dehydrogenase; transgenesis.

Introduction

Glucocorticoids are small lipophilic hormones that are synthesized in the adrenal gland and produce biologic effects in almost all tissues *(1)*. In humans and animals, glucocorticoids regulate carbohydrate and lipid metabolism, immune function, and stress responses. Because of their antiinflammatory and immunosuppressive properties, glucocorticoids are used widely as therapeutic agents. At the molecular level, glucocorticoids signal in target cells by binding to the glucocorticoid receptor (GR), which is a member of the ligand-dependent nuclear receptor family *(2)*. Glucocorticoids regulate both gene transcription and mRNA stability. Stimulatory effects of glucocorticoids on transcription are mediated by the binding of GR homodimers to glucocorticoid response elements in the promoter regions of many genes. By contrast, inhibitory effects of glucocorticoids on transcription are often due to direct protein-protein interactions of the GR with other transcription factors. (See refs. *2* and *3* for more detailed information on molecular mechanisms of glucocorticoid action.

Author to whom all correspondence and reprint requests should be addressed: Barbara E. Kream, Department of Medicine, MC-1850, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT. E-mail: kream@nso1.uchc.edu

Bimodal Effects of Glucocorticoids on Bone Formation

Cushing syndrome in humans is characterized by high circulating levels of glucocorticoids and vertebral fractures *(1,4,5)*. Similarly, humans and animals undergoing treatment with pharmacologic doses of glucocorticoids also exhibit bone loss and osteoporosis *(4,6–11)*. A distinguishing feature of glucocorticoid-induced osteoporosis in humans is decreased mean wall thickness of trabecular bone, which reflects a reduction in the amount of bone formed in each remodeling cycle *(12,13)*. Although the predominant consequence of chronically high levels of glucocorticoids is an impairment of bone formation, there is also an early increase in bone resorption after initiation of glucocorticoid therapy *(14)*.

Glucocorticoids decrease bone formation by both direct and indirect mechanisms (see refs. *6, 15,* and *16* for comprehensive reviews on this topic). Glucocorticoids signal in cells of the osteoblast lineage via classical GRs *(17–20)*. In rodent osteoblast-like cell cultures, high concentrations of glucocorticoids typically result in catabolic effects such as decreased protein, RNA, and DNA synthesis *(17,21–23)*. In calvarial organ cultures and cell cultures, high concentrations of glucocorticoid inhibit expression of type I collagen, the most abundant bone matrix protein that serves as a measure of osteoblastic bone formation *(24–31)*. Inhibitory effects of glucocorticoids on bone formation may be due to downregulation of insulin-like growth factor-1 (IGF-1) expression in osteoblasts *(16,32)*. However, glucocorticoids maintain their ability to inhibit collagen synthesis in fetal *Igf1* null calvariae, suggesting that glucocorticoids act in part by an IGF-1-independent pathway *(33)*. Glucocorticoids decrease cell replication in a variety of models: calvarial organ cultures, primary osteoblast cultures, and bone marrow stro-

mal cultures *(17,24,28,34–37)*. Chronic administration of glucocorticoid in mice decreases osteoblast generation in ex vivo bone marrow cultures *(11,38)*. Finally, glucocorticoids increase osteoblast and osteocyte apoptosis in vivo and in vitro *(11,39–42)*. Taken together, these data suggest that the pathogenesis of glucocorticoid-induced osteoporosis involves a decrease in bone formation owing to impairment of osteoblast renewal and function.

A long-standing paradox in bone biology is the disparate effects of glucocorticoids on osteoblast differentiation and bone formation in vivo and in vitro. Under specific experimental conditions, glucocorticoids are anabolic in bone organ and cell cultures; they are able to stimulate osteoblast differentiation, osteoblast function, and bone formation. Glucocorticoids increase the number of mineralized, bonelike nodules and the expression of osteoblast marker genes in rat osteoblast cultures grown in the presence of osteoinductive medium *(43–46)*. This anabolic effect is due to the proliferation and differentiation of glucocorticoid-dependent osteoprogenitors *(44,45)*. Likewise, the anabolic effect of glucocorticoids on osteoblast differentiation and the formation of mineralized nodules is observed in cultured bone marrow stromal cells from multiple species *(47–55)*.

Interestingly, some bone organ culture models display both the anabolic and catabolic effects of glucocorticoids on bone formation. In chick periosteum, the addition of dexamethasone to early cultures enhances osteoid formation, alkaline phosphatase activity, and replication of cells adjacent to the newly forming bone. However, the addition of dexamethasone to cultures after the onset of overt osteogenesis decreases alkaline phosphatase activity *(56,57)*. In rat calvarial organ cultures, glucocorticoids can both stimulate and inhibit collagen synthesis depending on the dose and duration of hormone treatment *(24,25)*; low concentrations of glucocorticoid cause an early stimulation of collagen synthesis, whereas higher concentrations are inhibitory at later time points. The early stimulation of collagen synthesis is mediated by IGFs and may represent enhancement of osteoblast differentiation *(58)*.

Genetic Models to Study the Role of Endogenous Glucocorticoids in Bone

As summarized, the effects of glucocorticoids on bone formation in vitro are model, time, and dose dependent. In general, pharmacologic concentrations of glucocorticoids inhibit cell proliferation, impair the function of more mature osteoblasts, and increase osteoblast and osteocyte apoptosis; the cited studies have provided mechanistic insight into the pathogenesis of glucocorticoid-induced osteoporosis. However, the relevance of the stimulatory effects of glucocorticoids on osteogenesis seen in culture models to a physiologic role of glucocorticoids in bone has not been established. Although data from culture models suggest that physiologic levels of glucocorticoids play a role in maintenance of the osteoblast phenotype and bone formation, this hypothesis needs to be tested in vivo. With the growing availability of molecular tools by which the mouse genome can be manipulated, it is possible to generate genetic models to test this hypothesis. As a means of determining the role of endogenous glucocorticoids in bone, we propose several loss-of-function mouse models in which disruption of glucocorticoid signaling in cells of the osteoblast lineage can be achieved. One model involves selectively ablating the GR gene (*Grl1*) or other components of the glucocorticoid signaling pathways in cells of the osteoblast lineage using Cre/loxP technology. Another model involves using a transgenic strategy to metabolically inactivate glucocorticoids in osteoblasts, leading to a disruption of downstream glucocorticoid signaling events.

Osteoblast-Specific Disruption of the GR in Mice

Genetic ablation of *Grl1* in mice by targeted mutagenesis in embryonic stem cells has been accomplished and could provide a model in which to examine the role of glucocorticoids in bone during development and postnatal life *(59)*. However, homozygous *Grl1* knockout mice show evidence of impaired embryonic development and failure to inflate their lungs at birth. This results in perinatal lethality, which precludes examination of the mice in postnatal life *(59,60)*. To circumvent the problem of perinatal lethality in this and other global gene knockout models, conditional gene disruption can be accomplished using Cre/loxP technology. Cre recombinase, an enzyme derived from bacteriophage P1, catalyzes the excision of a DNA fragment flanked by two 34-bp loxP sites *(61)*. To generate a tissue-specific gene knockout, two mouse lines are required, one with tissue-specific Cre expression and the second in which a portion of the gene of interest has been flanked with loxP sites. The two mouse lines are then interbred to create a tissue-specific knockout. Ideally, the introduction of loxP sites should not interfere with expression of the gene of interest, and expression of the Cre transgene should be spatially restricted to the tissue(s) of interest. If needed, there are now molecular tools to control additionally the temporal pattern of gene inactivation in mice using inducible promoters and ligand-activated Cre fusion proteins *(62)*.

To enable osteoblast-targeted gene ablation, we cloned Cre recombinase downstream of both a 2.3- and a 3.6-kb Col1a1 promoter fragment to produce Col2.3-Cre and Col3.6-Cre transgenes, respectively. Use of the chloramphenicol acetyltransferase and green fluorescent protein (GFP) reporter genes has revealed a differential pattern of Col2.3 and Col3.6 promoter expression in cells of the osteoblast lineage *(63,64)*. In bone marrow stromal and calvarial cell cultures derived from transgenic mice, Col2.3-GFP transgene expression occurs relatively late in osteoblast differentiation, along-

side expression of the differentiation markers bone sialoprotein and osteocalcin *(64)*. Moreover, Col2.3 expression is restricted to osteoblast cells localized in mineralizing nodules. By contrast, Col3.6-GFP is expressed early in these cultures in a more diffuse pattern, prior to expression of differentiated osteoblast markers *(64)*. Thus, the use of these promoter constructs allows us to target Cre expression to more differentiated osteoblasts or more broadly to osteoblasts and their stromal progenitor cells.

In preliminary experiments, Cre mRNA was expressed primarily in the long bone, calvariae, bone marrow, and tail of postnatal mice (unpublished results). To determine whether the Cre transgenes were active in vivo, Col2.3-Cre and Col3.6-Cre mice were interbred with ROSA26 Cre indicator mice, in which Cre-mediated excision of a loxP-flanked cassette in the ROSA locus results in constitutive expression of β-galactosidase to provide an indelible, historical marker of Cre expression *(65)*. In Cre+/ROSA26+ progeny, β-galactosidase expression was widespread in calvarial osteoblasts. With Col3.6-Cre, nearly all cells in calvariae were stained, whereas with Col2.3-Cre, there was absence of staining in suture cells. Thus, to produce GR ablation in cells of the osteoblast lineage, Col-Cre mice could be interbred with a mouse line having a loxP-flanked *Grl1* locus.

Recently, a mouse line has been developed in which exon 3 of *Grl1* has been flanked by loxP sites ($Grl1^{loxP}$) *(59,66)*. By crossing $Grl1^{loxP}$ mice with a transgenic strain expressing Cre in neuronal and glial cell precursors under the influence of the rat nestin (Nes) promoter and enhancer, mice with a disruption of *Grl1* in brain (GR^{NesCre}) were generated. GR^{NesCre} mice show symptoms of Cushing syndrome including behavioral changes, impairment of the hypothalamic-pituitary-adrenal axis, high circulating levels of glucocorticoids, and decreased bone mineral density *(66)*.

An osteoblast-targeted knockout of *Grl1* in mice has not yet been generated but would provide a potentially important model for assessing the role of glucocorticoids in bone during development and remodeling *(14)*. However, this model would not prevent glucocorticoids from signaling through alternative pathways such as the mineralocorticoid receptor (MR), which can bind glucocorticoids with high affinity *(67)*. MRs have been localized in human bone *(68,69)*, and functional MRs have been demonstrated in osteoblasts *(70)*. Because of the potential role of the MR in transducing a glucocorticoid signal, it would be desirable to block all glucocorticoid-signaling pathways in osteoblasts. To accomplish this, we have developed a strategy to inactivate glucocorticoid ligand in bone by driving transgenic expression of a metabolic enzyme, 11β-hydroxysteroid dehydrogenase-2 (11β-HSD2), in osteoblasts. As described subsequently, this model would recapitulate in bone a biologic paradigm evolved in the kidney to protect the MR from illicit activation by glucocorticoids.

Osteoblast-Targeted 11β-HSD2 to Disrupt Glucocorticoid Signaling in Bone

A paradox in the specificity of mineralocorticoid signaling arose when it was discovered that the MR was capable of binding both cortisol and aldosterone at high affinity in vitro. Given the high circulating levels of cortisol relative to aldosterone, how could a specific mineralocorticoid signal be transduced in the distal nephron and other mineralocorticoid target tissues? The answer was provided by the discovery of high levels of 11β-HSD activity in mineralocorticoid-responsive cells. 11β-HSD enzymes catalyze the interconversion of cortisol and cortisone in humans, and corticosterone and 11-dehydrocorticosterone in rodents *(71–73)*. In the kidney, the high-affinity NAD-dependent isoform known as 11β-HSD2 catalyzes the unidirectional conversion of the natural glucocorticoids cortisol and corticosterone to their inactive 11-keto metabolites cortisone and 11-dehydrocorticosterone, respectively, thereby metabolically protecting the MR from activation by glucocorticoids *(72)*. Mice with targeted inactivation of 11β-HSD2 show evidence of hypertension, hypotonic polyuria, and hypokalemia, symptoms that are observed in humans with the syndrome of apparent mineralocorticoid excess caused by mutations in the 11β-HSD2 gene *(74)*.

The 11β-HSD1 isoform, by contrast, is widely expressed in a number of tissues, including the liver, lung, adipose tissue, and central nervous system *(71,72)*. Biochemical analysis of 11β-HSD1 suggested that it could catalyze the bidirectional interconversion of cortisol and cortisone. However, recent studies have found that the reductase activity predominates in intact cells, perhaps as a result of its intracellular localization on the inner leaflet of the endoplasmic reticulum in close proximity to enzymes that generate the reduced cosubstrate NADPH. This suggests that the primary role of 11β-HSD1 is to convert cortisone to cortisol (or 11-dehydrocorticosterone to corticosterone), thereby enhancing access of the active metabolite to the GR. In mice, ablation of the 11β-HSD1 gene abolished the conversion of 11-dehydrocorticosterone to corticosterone in vivo, suggesting that 11β-HSD1 is the sole 11-reductase in the mouse *(75)*.

In recent years, there has been emerging data on the expression and function of endogenous 11β-HSD isoforms in bone. Expression of 11β-HSD1 has been reported in primary osteoblast cultures, in a number of osteoblastic cell lines, and in adult human bone osteoblasts *(76–78)*. The 11β-HSD1 isozyme is also present in osteoclasts *(77)*. 11β-HSD2 expression has been observed in osteosarcoma cell lines and in osteoblasts of human fetal bone *(76,77)*, whereas 11β-HSD2 immunoreactivity and mRNA were barely detectable in adult human bone *(77)*. In spite of the predominance of 11β-HSD1 in adult human bone, both 11-reductase and dehydrogenase activities were present. This observation

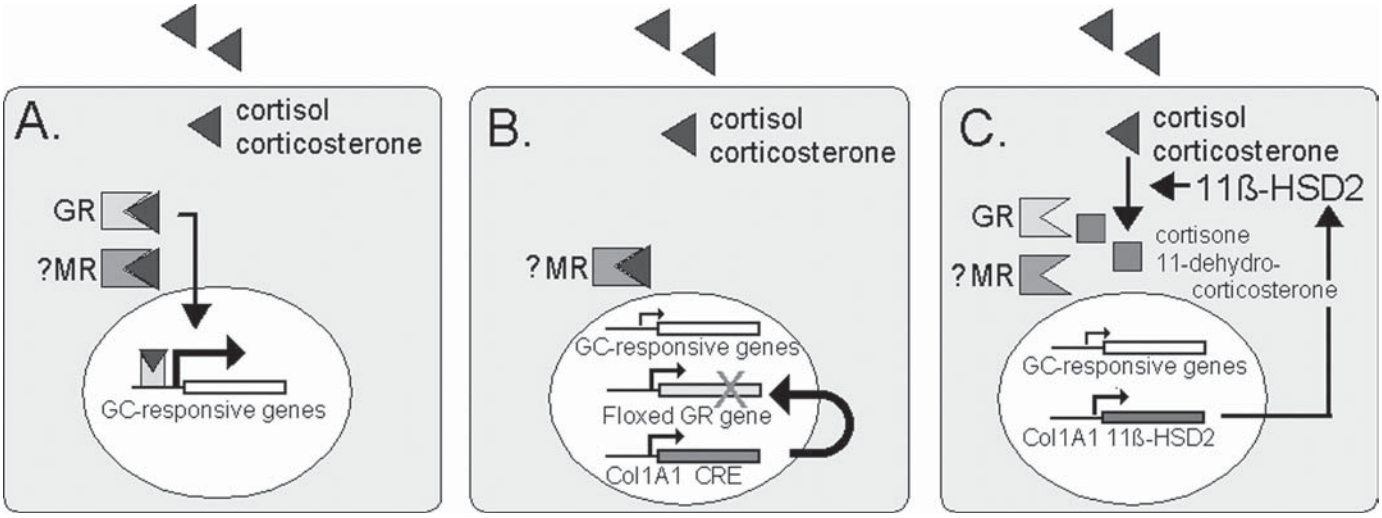

Fig. 1. Genetic loss-of-function strategies to determine the role of glucocorticoids in bone. (**A**) Regulation of glucocorticoid-responsive target genes in normal osteoblasts. (**B**) An osteoblast homozygous for a modified GR gene flanked by LoxP sites is shown. This cell also harbors a transgene driving expression of Cre recombinase under control of the Col1a1 promoter. The use of a relatively bone-specific Col1a1 promoter fragment results in selective inactivation of the GR gene in bone and a limited number of other tissues. Note that glucocorticoid signaling could still occur through the MR in this model. (**C**) An osteoblast with Col1a1-promoter-targeted overexpression of 11β-HSD2 is shown. In the presence of this enzyme, endogenous glucocorticoids will be rapidly converted to their biologically inactive 11-dehydro-metabolites. This strategy would preclude glucocorticoid signaling through both the GR and MR.

suggests that, in contrast to other tissues, 11β-HSD1 in human bone may display both 11-reductase and dehydrogenase activities. It is possible that this enzyme could function in the dual role of generating bioactive glucocorticoids locally or, alternatively, protecting the GR from excessive levels of glucocorticoids *(77)*. Recently, reciprocal regulation of 11β-HSD isoforms by cytokines was reported in MG-63 cells: interleukin-1 and tumor necrosis factor inhibited 11β-HSD2 expression and enhanced expression of 11β-HSD1, resulting in enhanced sensitivity to glucocorticoids *(79)*.

The metabolism of ligand by 11β-HSD2 serves as a mechanism for pre-receptor regulation of glucocorticoid signaling *(73)*. Transfection variants of rat osteosarcoma cells overexpressing 11β-HSD1 showed net conversion of cortisone to cortisol and decreased rates of cellular proliferation. By contrast, overexpression of 11β-HSD2 resulted in inactivation of cortisol to cortisone accompanied by enhanced proliferation *(80)*. Likewise, other steroid modifying enzymes are also gaining prominence as pre-receptor regulators of hormone action *(81)*. Thus, we reasoned that transgenic expression of 11β-HSD2 could provide a means to abrogate intracellular glucocorticoid signaling in osteoblasts, which have only low endogenous levels of the enzyme *(76)*. This strategy would afford the added advantage of preventing glucocorticoids from signaling through alternative pathways such as the MR, which can act as a functional GR in the absence of 11β-HSD2 (Fig. 1). Interestingly, there are high levels of the MR in developing bone, which show relatively low expression of 11β-HSD2 *(68,69)*. These observations raise the possibility that the MR may function primarily as a GR during bone development.

A first step in targeting 11β-HSD2 to bone was achieved using Col1a1 promoter fragments *(82)*. A rat 11β-HSD2 cDNA was cloned downstream of the 2.3- and 3.6-kb Col1a1 promoter fragments to produce Col2.3-HSD2 and Col3.6-HSD2, respectively. These constructs were transfected into osteoblastic ROS 17/2.8 and MC3T3-E1 cells, and expression of 11β-HSD2 mRNA and protein was confirmed by Northern blotting and immunohistochemistry, respectively *(82)*. Transfectants, which showed enzymatic activity of the transgene product, were refractory to glucocorticoid treatment. Inhibition of cell proliferation and regulation of osteoblast mRNA markers (repression of Col1a1 and osteocalcin, stimulation of bone sialoprotein) by glucocorticoids were blocked *(82)*. Moreover, ROS 17.2/8 cells cotransfected with a glucocorticoid-inducible promoter-reporter construct along with 11β-HSD2 showed greatly reduced cortisol- and corticosterone-dependent promoter induction relative to control cells. The effects of dexamethasone were only partially blocked in these studies because 11β-HSD2 is known to catalyze the bidirectional interconversion of dexamethasone and 11-dehydrodexamethasone *(71,72)*.

We have recently generated several transgenic lines of Col2.3-HSD2 mice by microinjection into CD-1 embryos (unpublished results). All produced normal-sized litters having the expected Mendelian ratio of wild-type and transgenic offspring. The transgene was highly expressed in calvariae, long bone, tendon, and tail, with little or no expression in other tissues. Interestingly, the level of targeted

transgene mRNA expression in bone was comparable with the level of endogenous 11β-HSD2 expression in the kidney, suggesting an appropriate level of expression to abrogate glucocorticoid responsiveness. Enzymatic activity of the 11β-HSD2 transgene was confirmed in calvarial organ cultures derived from Col2.3-HSD2 mice. Functional assays are underway to establish the efficacy of the 11β-HSD2 transgene in blocking glucocorticoid responses in vivo and ex vivo. The use of transgenic mice with osteoblast-targeted 11β-HSD2 should provide a novel in vivo glucocorticoid loss-of-function model in bone.

Conclusion

The role of endogenous glucocorticoids on osteoblast differentiation and function remains unclear. The classic genetic approach of disrupting the GR gene by homologous recombination results in perinatal lethality, limiting the utility of this model. To circumvent this problem, we have outlined a strategy for a tissue-selective knockout of the GR using Cre/loxP technology and Col1a1 promoter targeting. A second obstacle to a GR knockout is the possibility of redundant signaling pathways. Notably, the MR may transduce a glucocorticoid signal in tissues with low levels of endogenous 11β-HSD2 expression such as bone. We have therefore implemented a second strategy whereby 11β-HSD-2 expression is targeted to osteoblasts using Col1a1 promoter fragments. This approach, which mimics the paradigm of glucocorticoid metabolism in the kidney and other mineralocorticoid target tissues, will block glucocorticoid action by 11β-HSD2-catalyzed conversion of glucocorticoids to inactive metabolites in osteoblasts.

Together, these complementary loss-of-function strategies should allow elucidation of the effects of endogenous glucocorticoids on osteoblast differentiation, bone development, and bone remodeling. Moreover, phenotypic comparison of these models may provide new mechanistic insights into the role of the MR in bone. Other genetic models to consider include targeted disruption of the MR in bone, as well as targeted knockout of both 11β-HSD1 and 11β-HSD2. It would be interesting to determine whether overexpression of 11β-HSD1 in bone sensitizes bone to glucocorticoids in vivo, perhaps resulting in glucocorticoid-dependent osteopenia. Finally, ongoing studies on the expression patterns of Col1a1, osteocalcin, and other promoters during osteoblast differentiation should allow more selective targeting of gene disruption or overexpression at specific stages of osteoblast lineage progression.

Acknowledgments

We wish to thank Dr. Zygmunt Krozowski (Baker Medical Research Institute, Melbourne, Australia) for generously providing 11β-HSD2 cDNA and antibodies. Studies from our laboratory were supported by grants from the Deutsche Forschungsgemeinschaft (Wo 729/1-1) and from the National Institutes of Health (P01 AR38933).

References

1. Orth, D. N. and Kovacs, W. J. (1998). In: *Williams textbook of endocrinology, 9th ed.* Foster, D. W., Kronenberg, H. M., and Larsen, P. R. (eds.). W.B. Saunders: Philadelphia.
2. Beato, M., Herrlich, P., and Evans, R. M. (1995). *Cell* **83,** 851–857.
3. Beato, M., Chavez, S., and Truss, M. (1996). *Steroids* **61,** 240–251.
4. Lukert, B. P. and Raisz, L. G. (1994). *Adv. Endocrinol. Metab.* **5,** 97–135.
5. Cushing, H. (1932). *Bull. Johns Hopkins Hosp.* **50,** 137–195.
6. Lukert, B. P. and Kream, B. E. (1996). In: *Principles of bone biology.* Bilezikian, J., Raisz, L. G., and Rodan, G. A. (eds.). Academic: San Diego.
7. Altman, A., Hockberg, Z., and Silbermann, M. (1992). *Calcif. Tissue Int.* **51,** 298–304.
8. Quarles, L. D. (1992). *Am. J. Physiol.* **263,** E136–E141.
9. Turner, R. T., Hannon, K. S., Greene, V. S., and Bell, N. H. (1995). *Calcif. Tissue Int.* **56,** 311–315.
10. Ortoft, G., Bruel, A., Andreassen, T. T., and Oxlund, H. (1995). *Bone* **17,** 543–548.
11. Weinstein, R. S., Jilka, R. L., Parfitt, A. M., and Manolagas, S. C. (1998). *J. Clin. Invest.* **102,** 274–282.
12. Dempster, D. W., Arlot, M. A., and Meunier, P. J. (1983). *Calcif. Tissue Int.* **35,** 410–417.
13. Dempster, D. W. (1989*). J. Bone Miner. Res.* **4,** 137–141.
14. Canalis, E. and Giustina, A. (2001). *J. Clin. Endocrinol. Metab.* **86,** 5681–5685.
15. Manolagas, S. C. and Weinstein, R. S. (1999). *J. Bone Miner. Res.* **14,** 1061–1066.
16. Canalis, E. (1996). *J. Clin. Endocrinol. Metab.* **81,** 3441–3447.
17. Chen, T. L., Aronow, L., and Feldman, D. (1977). *Endocrinology* **100,** 619–628.
18. Manolagas, S. C. and Anderson, D. C. (1978). *J. Endocrinol.* **76,** 379,380.
19. Haussler, M. R., Manolagas, S. C., and Deftos, L. J. (1980). *Biochem. Biophys. Res. Commun.* **94,** 373–380.
20. Abu, E. O., Horner, A., Kusec, V., Triffitt, J. T., and Compston, J. E. (2000). *J. Clin. Endocrinol. Metab.* **85,** 883–889.
21. Peck, W. A., Brand, J., and Miller, I. (1967). *Proc. Natl. Acad. Sci. USA* **57,** 1599–1606.
22. Choe, J., Stern, P., and Feldman, D. (1978). *J. Steroid Biochem.* **9,** 265–271.
23. Wong, G. L. (1979). *J. Biol. Chem.* **254,** 6337–6440.
24. Dietrich, J. W., Canalis, E. M., Maina, D. M., and Raisz, L. G. (1978). *Endocrinology* **104,** 715–721.
25. Canalis, E. (1983). *Endocrinology* **112,** 931–939.
26. Hahn, T. J., Westbrook, S. L., and Halstead, L. R. (1984). *Endocrinology* **114,** 1864–1870.
27. Kream, B. E., Petersen, D. N., and Raisz, L. G. (1990). *Endocrinology* **126,** 1576–1583.
28. Hodge, B. O. and Kream, B. E. (1988). *Endocrinology* **122,** 2127–2133.
29. Ng, K. W., Manji, S. S., Young, M. F., and Findlay, D. M. (1989). *Mol. Endocrinol.* **3,** 2079–2085.
30. Kim, H. T. and Chen, T. L. (1989). *Mol. Endocrinol.* **3,** 97–104.
31. Delany, A. M., Gabbitas, B. Y., and Canalis, E. (1995). *J. Cell. Biochem.* **57,** 488–494.
32. McCarthy, T. L., Centrella, M. , and Canalis, E. (1990). *Endocrinology* **126,** 1569–1575.
33. Woitge, H. W. and Kream, B. E. (2000*). J. Bone Miner. Res.* **15,** 1956–1964.
34. Canalis, E. (1984). *Calcif. Tissue Int.* **36,** 158–166.
35. Chyun, Y. S., Kream, B. E., and Raisz, L. G. (1984). *Endocrinology* **114,** 477–480.
36. Hughes-Fulford, M., Appel, R., Kumegawa, M., and Schmidt, J. (1992). *Exp. Cell. Res.* **203,** 150–156.

37. Walsh, S., Jordan, G. R., Jefferiss, C., Stewart, K., and Beresford, J. N. (2001). *Rheumatology (Oxford)* **40,** 74–83.
38. Simmons, D. J., Kidder, L., and Thomas, M. (1990). *Calcif. Tissue Int.* **46,** 327–332.
39. Gohel, A., McCarthy, M. B., and Gronowicz, G. (1999). *Endocrinology* **140,** 5339–5347.
40. Silvestrini, G., Ballanti, P., Patacchioli, F. R., Mocetti, P., Di Grezia, R., Wedard, B. M., Angelucci, L., and Bonucci, E. (2000). *Bone* **26,** 33–42.
41. Plotkin, L. I., Weinstein, R. S., Parfitt, A. M., Roberson, P. K., Manolagas, S. C., and Bellido, T. (1999). *J. Clin. Invest.* **104,** 1363–1374.
42. Weinstein, R. S., Nicholas, R. W., and Manolagas, S. C. (2000). *J. Clin. Endocrinol. Metab.* **85,** 2907–2912.
43. Bellows, C. G., Aubin, J., and Heersche, J. N. M. (1987). *Endocrinology* **121,** 1985–1992.
44. Bellows, C. G. and Aubin, J. E. (1989). *Dev. Biol.* **133,** 8–13.
45. Bellows, C. G., Heersche, J. N. M., and Aubin, J. E. (1990). *Dev. Biol.* **140,** 132–138.
46. Shalhoub, V., Conlon, D., Tassinari, M., Quinn, C., Partridge, N., Stein, G. S., and Lian, J. B. (1992). *J. Cell. Biochem.* **50,** 425–440.
47. McCulloch, C. A., Strugurescu, M., Hughes, F., Melcher, A. H., and Aubin, J. E. (1991). *Blood* **77,** 1906–1911.
48. Kasugai, S., Todescan, R., Nagata, T., Yao, K., Butler, W. T., and Sodek, J. (1991). *J. Cell. Physiol.* **147,** 111–120.
49. Kamalia, N., McCulloch, C. A. G., Tenebaum, H. C., and Limeback, H. (1992). *Blood* **79,** 320–326.
50. Cheng, S., Yang, J. W., Rifas, L., Zhang, S., and Avioli, L. (1994). *Endocrinology* **134,** 277–286.
51. Rickard, D. J., Sullivan, T. A., Shenker, B. J., Leboy, P. S., and Kazhdan, I. (1994). *Dev. Biol.* **161,** 218–228.
52. Malaval, L., Modrowski, D., Gupta, A. K., and Aubin, J. E. (1994). *J. Cell. Physiol.* **158,** 555–572.
53. Herbertson, A. and Aubin, J. E. (1995). *J. Bone Miner. Res.* **10,** 285–294.
54. Cheng, S. L., Zhang, S. F., and Avioli, L. V. (1996). *J. Cell. Biochem.* **61,** 182–193.
55. Aubin, J. E. (1999). *J. Cell. Biochem.* **72,** 396–410.
56. Tenenbaum, H. C. and Heersche, J. N. M. (1985). *Endocrinology* **117,** 2211–2217.
57. McCulloch, C. A. G. and Tenenbaum, H. C. (1986). *Anat. Rec.* **215,** 397–402.
58. Kream, B. E., Tetradis, S., Lafrancis, D., Fall, P. M., Feyen, J. H., and Raisz, L. G. (1997). *J. Bone Miner. Res.* **12,** 889–895.
59. Tronche, F., Kellendonk, C., Reichardt, H. M., and Schutz, G. (1998). *Curr. Opin. Genet. Dev.* **8,** 532–538.
60. Reichardt, H. M., Tronche, F., Berger, S., Kellendonk, C., and Schutz, G. (2000). *Adv. Pharmacol.* **47,** 1–21.
61. Nagy, A. (2000). *Genesis* **26,** 99–109.
62. Lewandoski, M. (2001). *Nat. Rev. Genet.* **2,** 743–755.
63. Dacic, S., Kalajzic, I., Visnjic, D., Lichtler, A. C., and Rowe, D. W. (2001). *J. Bone Miner. Res.* **16,** 1228–1236.
64. Kalajzic, I., Kalajzic, Z., Kaliterna, M., Gronowicz, G., Clark, S. H., Lichtler, A. C., and Rowe, D. (2002). *J. Bone Miner. Res.* **17,** 15–25.
65. Soriano, P. (1999). *Nat. Genet.* **21,** 70,71.
66. Tronche, F., Kellendonk, C., Kretz, O., Gass, P., Anlag, K., Orban, P. C., Bock, R., Klein, R., and Schutz, G. (1999). *Nat. Genet.* **23,** 99–103.
67. Funder, J. W. (1992). *J. Steroid Biochem. Mol. Biol.* **43,** 389–394.
68. Condon, J., Gosden, C., Gardener, D., Nickson, P., Hewison, M., Howie, A. J., and Stewart, P. M. (1998). *J. Clin. Endocrinol. Metab.* **83,** 4490–4497.
69. Beavan, S., Horner, A., Bord, S., Ireland, D., and Compston, J. (2001). *J. Bone Miner. Res.* **16,** 1496–504.
70. Agarwal, M. K., Mirshahi, F., Mirshahi, M., Bracq, S., Chentoufi, J., Hott, M., Jullienne, A., and Marie, P. J. (1996). *Am. J. Physiol.* **270,** C1088–C1095.
71. Krozowski, Z., Li, K. X., Koyama, K., Smith, R. E., Obeyesekere, V. R., Stein-Oakley, A., Sasano, H., Coulter, C., Cole, T., and Sheppard, K. E. (1999). *J. Steroid Biochem. Mol. Biol.* **69,** 391–401.
72. Krozowski, Z. (1999). *Mol. Cell. Endocrinol.* **151,** 121–127.
73. Sandeep, T. C. and Walker, B. R. (2001). *Trends Endocrinol. Metab.* **12,** 446–453.
74. Kotelevtsev, Y., Brown, R. W., Fleming, S., Kenyon, C., Edwards, C. R., Seckl, J. R., and Mullins, J. J. (1999). *J. Clin. Invest.* **103,** 683–689.
75. Kotelevtsev, Y., Holmes, M. C., Burchell, A., Houston, P. M., Schmoll, D., Jamieson, P., Best, R., Brown, R., Edwards, C. R., Seckl, J. R., and Mullins, J. J. (1997). *Proc. Natl. Acad. Sci. USA* **94,** 14,924–14,929.
76. Bland, R., Worker, C. A., Noble, B. S., Eyre, L. J., Bujalska, I. J., Sheppard, M. C., Stewart, P. M., and Hewison, M. (1999). *J. Endocrinol.* **161,** 455–464.
77. Cooper, M. S., Walker, E. A., Bland, R., Fraser, W. D., Hewison, M., and Stewart, P. M. (2000). *Bone* **27,** 375–381.
78. Eyre, L. J., Rabbitt, E. H., Bland, R., Hughes, S. V., Cooper, M. S., Sheppard, M. C., Stewart, P. M., and Hewison, M. (2001). *J. Cell. Biochem.* **81,** 453–462.
79. Cooper, M. S., Bujalska, I., Rabbitt, E., Walker, E. A., Bland, R., Sheppard, M. C., Hewison, M., and Stewart, P. M. (2001). *J. Bone Miner. Res.* **16,** 1037–1044.
80. Rabbitt, E. H., Lavery, G. G., Walker, E. A., Cooper, M. S., Stewart, P. M., and Hewison, M. (2002). *FASEB J.* **16,** 36–44.
81. Song, W. C. and Melner, M. H. (2000). *Endocrinology* **141,** 1587–1589.
82. Woitge, H., Harrison, J., Ivkosic, A., Krozowski, Z., and Kream, B. (2001). *Endocrinology* **142,** 1341–1348.