A NEW CONCEPT OF TREATMENT FOR OSTEOPOROSIS: EXPLOITING THE FUNCTION OF PARATHYROID HORMONE

Doo-Hong Park[1], Eun-Kyeong Jung[1], Dong-Soo Jang[1], Eun-Kyoung Lee[1], Hyung-Guen Lim[2], Kyung-Hwan Jung[2], Bon-Heun Koo[3], Kwang-Hoe Chung[3], Hong Mo Moon, and Seung Kil Lim[4]

Labs of Prokaryotic Gene Expression[1], Fermentation[2], and Protein[3], Mogam Biotechnology Research Institute, Yongin-si, Kyonggi-do 449-910, and Department of Internal Medicine, College of Medicine, Yonsei University[4], Seoul, Korea

INTRODUCTION

Skeleton of human body performs essential functions in life; it protects internal organs from injury, provides a framework for the body, serves as a mineral reservoir for the mineral homeostasis, and permits locomotion. Thus, any problem with this human organ causes profound effect on the quality of human life and osteoporosis is the major problem so far known. Osteoporosis is a metabolic bone disease that is characterized by reduced amount of bone mass and deteriorated bone micro-architecture. This disease causes decreased physical strength of the skeleton, consequently increasing susceptibility to fractures. Osteoporosis has a worldwide prevalence. 30-40% of postmenopausal women, and the elderly persons at the age of 70 or older will experience at least one osteoporotic fracture during their lives (1). The annual fracture rate due to osteoporosis in the United States is estimated to be 1.3 million, including 300,000 new cases of osteoporotic hip fractures (2). The impact of osteoporosis on the health care problem is well appreciated; as menifested in the statistics, six months after a hip fracture only 25% of patients are fully recovered, whereas 50% need assistance even for routine daily activities and 25% rely on long-term nursing care (3). This inflicts annual health care cost of over 10 billion dollars on the United States alone. It is obvious that as the aged population increases, the incidence of osteoporosis-related bone fractures will also rise.

In recent years there have been wider interests and higher investments on the bone and mineral research, starting to unveil the underlying mechanisms for the pathogenesis of osteoporosis. Although it is yet far from the complete understanding of the disease, accumulation of knowledge in the field of bone and mineral research will undoubtedly lead to more effective measures to deal with osteoporosis, both preventive and therapeutic.

BONE AND BONE REMODELING

Bone is chemically composed of two main parts, organic matrix and minerals, and anatomically comprised of 80% of cortical bone and 20% of trabecular cancellous bone. Cortical bone is compact and surrounds cancellous bone, contributing to structural integrity. Trabecular bone is most commonly found in the vertebrae, the pelvis and the ends of the long bones. It is metabolically more active than cortical bone and thus more responsive to skeletal changes including remodeling signals. It is composed of interconnecting lattice with an architecture designed to resist mechanical loads. The effect of osteoporosis is much more significant on trabecular bone than on cortical bone, as evidenced by the fact that osteoporotic fractures in the long bones tend to be in the proximal femur and distal radius which have greatest proportion of cancellous bone.

There are four major types of bone cells: osteoblasts, osteoclasts, osteocytes, and bone lining cells. Osteoblasts are derived from mesenchymal cells (4,5). They produce the bone matrix proteins including type I collagen, osteocalcin, osteonectin and others to form the organic bone matrix termed osteoid (6). Osteoblasts are also responsible for the mineralization of osteoid to complete the bone formation process (7,8). These bone cells are primary target for the actions of growth factors, cytokines, and hormones that are known to be involved in the regulation of bone metabolism (2,9-17). They include insulin-like growth factors, fibroblast growth factor, transforming growth factor-β, prostaglandin E, interleukin-1, parathyroid hormone, estradiol, and 1,25-dihydroxyvitamin D. Hence, osteoblasts serve as the major regulatory site in bone homeostasis. Osteoclasts are multinucleated cells which are formed by the fusion of mononuclear hematopoietic precursors of monocyte-macrophage lineage (18,19).

This process is controlled by such factors as macrophage colony stimulating factor and $pp60^{c\text{-}src}$, as revealed from the studies of naturally occuring mutant (20-25) and gene knock-out animals (26-28). Hormones such as calcitonin, parathyroid hormone and 1,25-dihydroxyvitamin D, that play major roles in the calcium homeostasis, are also involved in the regulation of the development of osteoclasts from their precursors (24,29-34). Mature and functional osteoclasts are characterized biochemically as rich in tartrate-resistant acid phosphatase and morphologically having ruffled border and clear zone or sealing zone (35). The ruffled border is a complex structure of deeply infolded finger-like plasma membranes adjacent to the bone surface. The bone-resorbing area under the ruffled border maintains acidic microenvironment suitable for bone resorption. Protons generated in osteoclasts by carbonic anhydrase II are secreted into the resorbing area by proton pumps present in ruffled border membranes. Lysosomal proteolytic enzymes are also secreted into the resorbing area to degrade the organic matrix of bone. The ruffled border is surrounded by the clear zone where tight attachment of osteoclasts to the bone surface takes place probably via interaction between integrins on the osteoclast membrane and their Arg-Gly-Asp-containing matrix proteins on the bone surface (36,37). Osteocytes are osteoblasts that are internalized in lacunae, deep in bone. Although osteocytes do not divide, they are believed to take part in bone metabolism. They may contribute to mineral transportation (38). Osteocytes may detect local mechanical loading and transmit signals to the surface osteoblasts and bone lining cells, which initiate either bone formation or resorption (38,39). Bone-lining cells are flattened cells lining the endosteal surfaces and trabeculae. These cells share some properties with osteoblasts, including expression of hormone receptors (6,40).

Bone remodeling is a lifelong renewal process of bone by which the structural and mechanical integrity of the skeleton is preserved. This also plays a role in the calcium homeostasis. Each year 10-30% of the adult bone is replaced by remodeling. Normal bone remodeling proceeds in a highly regulated cycle that involves a complex interplay of systemic hormones, local growth factors and cytokines, and mechanical stimuli (41,42). The remodeling process consists of four distinct events: activation, resorption, reversal, and formation (42). In the activation stage, the bone surface is exposed as bone-lining cells degrade the bone matrix proteins and then retract from the surface. New osteoclasts are differentiated from their precursors and mature cells are activated to start resorption. During the resorption period the stimulated multinucleated osteoclasts erode bone and form a cavity called resorption lacunae. Each osteoclast cell can resorb bone over an area two to three times larger than itself. The resorption takes place over one to three weeks. Although the mechanisms which control the extent and termination of resorption are not clearly defined, a number of possibilities have been postulated. Number and limited life span of osteoclasts and inactivation of osteoclasts by accumulation of calcium or transforming growth factor-β in the resorbing area may account for the regulation. During the reversal period, osteoclasts disappear and osteoblasts are attracted to the resorbed area. In the formation stage, the newly arrived osteoblasts deposit osteoid which is mineralized after five to ten days. The bone formation process is controlled by both systemic and local factors. As the formation process ends, osteoblasts change shape from columnar, densely packed structures to flatter, broader cells and eventually become bone-lining cells. The formation process takes about three months, but mineralization continues for another three to six months before the bone reaches maximum density.

PATHOPHYSIOLOGY AND THERAPIES OF OSTEOPOROSIS

Osteoporosis is a disease resulting from a disturbance in the normal balance between bone resorption and bone formation. The disease is categorized into two groups depending on the cause of the disturbance: the primary osteoporosis that is mainly caused by the loss of gonadal function and aging, and the secondary osteoporosis which is associated with other specific diseases (43). Osteoporosis due to the loss of gonadal function, especially in postmenopausal women, is termed type I, whereas that associated with aging is termed type II. Bone density of human being peaks at around 30 years of age and starting from 40-50 years of age, both men and women lose bone at a rate of 0.3 to 0.5 % per year (1,44). The rate abruptly increases as high as 10-fold after menopause. This increase is associated with the increases of both bone resorption and formation, with the former exceeding the latter, and an increase in the number of osteoclasts in trabecular bone. The pathogenetic role of estrogen in the postmenopausal ostoeporosis has been clearly demonstrated (2,43-46). The mechanism by which estrogens exert their effect on suppressing the osteoclast development has recently been suggested as mediated via regulation of interleukin-6 expression (47). In accordance with this hypothesis, ovariectomy did not induce any change in either bone mass or bone

remodeling rates in interleukin-6 deficient mice generated by gene targeting (48). In type II 'senile' osteoporosis, the bone loss is associated with declined number and activity of osteoblasts. Although the mechanism for this phenomenon is not yet known, it was shown that the number of osteoblast colony forming units formed in marrow cultures from mice with accelerated scenescence dramatically decreased, as compared to that from normal mice (49). This suggests that development of mature osteoblasts from their marrow precursors in mice may be impaired with aging. Other changes associated with aging, such as increased level of parathyroid hormone, decreased calcium absorption possibily due to reduced level of 1,25-dihydroxyvitamin D, and reduced level of growth hormone have also been implicated in the pathogenesis of type II osteoporosis (44,50-52). Another difference between postmenopausal and senile bone losses is that the former primarily occurs in trabecular bone, whereas the latter in cortical bone.

Therapies for osteoporosis can be divided into two categories, one that inhibits bone resorption and the other that stimulates bone formation. Drugs of the former category can prevent further bone loss and hence are most appropriate for prophylactic purposes. One major drawback, however, of these drugs is that they are of little use for the patients with 'established osteoporosis' where bone density has fallen below the fracture threshold. Unfortunately, the most current osteoporosis drugs fall into this category, which is represented by estrogens, calcitonin, and bisphosphonates (3).

Estrogens are the cheapest but still one of the most effective drugs for osteoporosis. Since the study by Lindsay *et al.* (53), demonstrating the excellent efficacy of estrogen in preserving the bone density of postmenopausal osteoporosis patients without further bone loss, there have been numerous studies to elucidate the mechanism underlying the estrogen action (2,43-46). Although estrogen therapy is the treatment of choice for prevention of postmenopausal osteoporosis, more than 50% of patients prescribed for estrogen give up the medication due to serious side effects such as increased risk of breast and uterine cancers and resumption of menstrual bleeding. Development of analogues of estrogen, such as raloxifene, is underway to reduce the side effects (54,55). Raloxifene acts as an agonist of estrogen in bone cells but as an antagonist in the reproductive tissues (56).

Calcitonin is an alternative therapy in the prevention of postmenopausal bone loss in women who cannot or will not take estrogens. It is a peptide of 32 amino acid residues, in which the carboxy-terminal amidation is essential for the bioactivity of the peptide (57). Calcitonin acts directly on osteoclasts and their precursors through specific receptor present on the cells of osteoclast lineage. Both differentiation of precursors into mature osteoclasts and resorbing activity of the mature osteoclasts are inhibited by calcitonin. This is a relatively expensive therapy and there appears to be development of a gradual resistance to calcitonin associated with long-term use, leading to reduced efficacy of the therapy.

Bisphosphonates are analogues of pyrophosphate that has been implicated as a physiological regulator of calcification and decalcification. These drugs decrease bone resorption by becoming adsorbed onto bone crystal (58). Bisphosphonates also appear to have effects on the number and activity of osteoclasts. They inhibit the formation of mature osteoclasts from their precursors in long-term bone marrow culture (59). The bone-resorbing activity of osteoclasts is inhibited *in vitro* by bisphosphonates (60). The excellent *in vivo* stability and continuous administration for more than six months of these drugs may cause such side effects as impaired bone mineralization and inhibition of microfracture repair. Second and third generations of bisphophonates with reduced side effects and improved efficacy are currently being evaluated in several clinical trials (2,61).

Several new drugs that are based on the underlying mechanisms for bone resorption are being developed. These include: inhibitors of Src protein tyrosine kinase in osteoclasts that can block formation of functional bone-resorbing cells; antagonists for integrin $\alpha_v\beta_3$ of osteoclasts that inhibit the attachment of functional osteoclasts to bone surface; inhibitors for carbonic anhydrase II and H^+ ATPase that block production and secretion of hydrogen ions, respectively; and inhibitors for proteases of osteoclasts, such as Cathepsins L and K, that block the degradation of bone matrix proteins by these enzymes (62-68). Most of these drugs are in early stage of development and thus should await the verification of their efficacy in human trials.

It is obvious that ideal therapy for osteoporosis can actually restore the bone lost from the disease. In this case, it is mandatory that the newly formed bone should be similar in architecture to the normal bone. Several drugs that may fulfill these requirements are under extensive tests in clinical human trials, among which fluoride and parathyroid hormone are the front runners.

Fluoride stimulates proliferation and differentiation of osteoblasts (69). A clinical study by Riggs *et al.* (70) in osteoporosis using a high dosage of 75 mg NaF/day for 4 years showed a

remarkable increase in the vertebral bone density as compared to placebo-treated group. This increase in bone density, however, was not accompanied by improvements in incidence of fractures or percentage of patients remaining fracture free, suggesting quality of the bone restored by fluoride might not be the same as that of normal bone. The biphasic effect of fluoride on bone structure and mechanical strength has been well documented in animal studies that high fluoride dosage at over 200 mg/l of drinking water reduces mechanical strength of bone, whereas lower fluoride administration of less than 75 mg/l improves or does not affect mechanical strength (71). Attempts have been made to reduce the deleterious effect on bone quality while maintaining the beneficial effects of fluoride by using slow-releasing NaF, intermittent withdrawal of fluoride treatment, and providing calcium citrate for optimal mineralization of newly formed bone matrix (72).

Development of parathyroid hormone as a promising new therapy that can actually increase the bone density while maintaining the mechanical strength will be discussed below in more detail.

PARATHYROID HORMONE : A NEW CONCEPT OF THERAPY FOR OSTEOPOROSIS

Parathyroid hormone (PTH) is a peptide of 84 amino acid residues synthesized in and secreted from parathyroid gland. It is a major regulator of calcium and skeletal homeostases, acting primarily through its receptor on target cells in bone and kidney. PTH has been traditionally considered to be physiologically catabolic for bone. It induces mobilization of calcium from bone by stimulating bone resorption. The catabolic effect of PTH on bone appears to be indirect, since, in *in vitro* resorption assay, coculture of primary osteoblasts or osteoblast-like cell lines with osteoclasts is necessary for osteoclasts to respond to PTH in the increase of bone resorption (73). In fact, cDNA clones for the PTH receptors have also been isolated from rat osteoblast-like osteosarcoma cells using expression cloning technique (74). cDNAs that encode PTH receptors have been cloned from several sources, including opossum kidney and human renal and bone tissues (75-77). These receptors have been shown to bind both PTH and PTH-related peptide (PTHrP), and hence termed PTH/PTHrP receptor. Analysis of primary sequences deduced from the nucleotide sequences of cDNA clones for the PTH/PTHrP receptors suggests the existence of 10 hydrophobic domains, 7 of which are predicted to be membrane-spanning. These receptors represent the first members of a new subfamily of G protein-linked receptor, which transmit signals through both adenylate cyclase and phospholipase C. Studies of structure and function have defined the amino terminal domain ranging from residues 1 through 34 of PTH (PTH(1-34)) is necessary and sufficient for full biological activity of full-length PTH (PTH(1-84)) (78,79). Binding of PTH(1-84) or PTH(1-34) to its receptor can activate both adenylate cyclase and phospholipase C in the same cell (80). The amino-terminal residues are crucial for activation of adenylate cyclase, since deletion of these residues dramatically reduces the activation of adenylate cyclase but maintains the stimulating activity of phospholipase C (79-83).

The non-physiological activity of PTH on bone metabolism, that is stimulation of bone formation by exogenous administration of PTH intermittently at low doses, has mainly been demonstrated in *in vivo* animal studies (84-92). Rats with osteopenia induced by ovariectomy share many similar characteristics with patients with postmenopausal osteoporosis. These include: increased bone turnover with resorption exceeding formation; an initial rapid phase of bone loss followed by a much slower phase; greater loss of cancellous than cortical bone; decreased absorption of calcium; and similar skeletal response to therapy with estrogen, bisphosphonates, calcitonin, PTH, and exercise. All of early studies were performed using parathyroid extract, whereas most recent data were obtained using synthetic PTH(1-34) due to limited availability of the full-length PTH. Recent success in overexpression of PTH(1-84) using recombinant DNA technology enabled use of the full-length PTH in the animal studies (93-97).

The histomorphometric evaluations showed the positive effects of daily injection of human PTH (hPTH) on such parameters as cancellous bone volume, bone formation rate, mineral apposition rate, mineralizing surface, and trabecular thickness, demonstrating the anabolic activity of hPTH(1-84) and the amino-terminal fragments of hPTH. The bone formed by the intermittent administration of hPTH is likely to be of normal quality as assessed by biomechanical testing of such parameters as maximum load, stress, and stiffness (92,98,99). Similar effects were observed not only with ovariectomized rats but also with senile rats (99). Taken together, these results confirm that daily injection of hPTH can restore bone, especially trabecular bone, lost either from menopause or from aging without sacrificing the bone quality. The anabolic effect of hPTH on bone has been compared to those of estrogen and

bisphosphonates, all proving the better effectiveness of hPTH (91,92). It has been reported that bone mass restored by PTH may decrease rapidly upon discontinuation of PTH injection (100). This rapid bone loss, however, could be prevented by physical exercise or antiresorptive agents such as estrogen and bisphosphonates, which were shown to maintain the PTH-restored bone mass even after the cessation of PTH administration (101,102). The concept of combined or cyclic treatment with PTH and an antiresorptive agent has been evaluated using estrogen, bisphosphonates or calcitonin as the antiresorptive agent (102-105). Some of these regimens resulted in a greater improvement in cancellous bone mass than with PTH alone, especially during the rapid bone loss phase.

The anabolic effects of PTH on bone demonstrated in animal studies have led to the human trials of hPTH administration in osteoporotic patients, both men and women (106-108). All the data available from human studies were done with the amino-terminal fragments of hPTH (hPTH(1-34) or hPTH(1-38)). A few clinical studies using hPTH(1-84) are underway and thus the results will be available shortly. Results of small multicenter trial using daily injections of 500 U of synthetic hPTH(1-34) for 6-24 months showed striking increases in new bone accretion, cancellous bone volume, and osteoid covered cancellous surfaces (109). These effects were not accompanied with net gain of internal calcium absorption, suggesting the increase in cancellous bone volume might be obtained at the expense of cortical bone loss. This concern led to the use of hPTH in combination with 1,25-dihydroxyvitamin D or other antiresorptive agents such as estrogen and calcitonin (110,111). Most of the combination therapies resulted in increase of volume and density in cancellous bone as in the monotherapy with PTH but without any detrimental effects on cortical bone. Effectiveness of intermittent administration of PTH in the prevention of bone loss due to estrogen deficiency was demonstrated in women with endometriosis who were being treated with nafarelin (an analogue of gonadotropin-releasing hormone) (112).

The mechanism whereby intermittent PTH therapy exerts anabolic effects on bone is not currently defined. Possible involvement of insulin-like growth factor I (IGF-I) in the anabolic action of PTH was first suggested by Canalis *et al.* (113) showing in the fetal rat calvaria model that only intermittent but not continuous treatment of PTH increased local production of IGF-I. This hypothesis was further supported by Watson *et al.* (114), who used *in situ* hybridization techniques to show that restoration of bone mass by PTH was associated with increased osteoblast IGF-I gene expression. Of the two signal transduction pathways, adenylate cyclase and phospholipase C, stimulated by PTH, the former pathway is likely to be responsible for the *in vivo* bone formation activity of PTH, as demonstrated by Rixon *et al.* (115), who showed in a study using ovariectomized rats that analogs of PTH that stimulated adenylate cyclase pathway were able to increase bone mass, whereas fragments that only activated phospholipase C pathway failed to do so.

DEVELOPMENT OF RECOMBINANT HUMAN PARATHYROID HORMONE AS A THERAPEUTIC AND PREVENTIVE DRUG FOR OSTEOPOROSIS

The ability of PTH that stimulates restoration of bone of normal quality, as demonstrated in many animal and human studies, have led many drug companies to the development of hPTH as an ideal management for osteoporosis, both preventive and therapeutic. Two forms of hPTH, hPTH(1-34) and hPTH(1-84), are being tested in human trials. We are currently developing the full-length hPTH using recombinant DNA technology. As stated above, the amino-terminal residues of PTH are critical in transmitting signal to the adenylate cyclase pathway and this signaling is likely indispensible for the anabolic action of the peptide. Processing of the amino-terminal methionine of foreign proteins overexpressed in *E. coli* is often incomplete, leaving unprocessed protein with an additional methionine at the amino terminus. The amino-terminal methionine can be removed *in vitro* by using methionyl aminopeptidase. This reaction, however, often results in either a mixture of properly processed and unprocessed proteins or proteins with unwanted modification such as deamidation of asparagine residue, all causing problems in later steps of purification. In addition, the size of peptides such as hPTH(1-84) is in many cases not large enough for efficient expression in *E. coli*. These properties of hPTH(1-84) led us to the development of a fusion expression strategy for efficient and economic expression of the peptide in *E. coli*. In choosing a fusion partner for hPTH(1-84), two points were considered, effectiveness in aiding expression of the target protein and ease and efficiency of cleavage of the target protein from fusion partner. In terms of the expression efficiency, we maximized the expression level of hPTH(1-84) by using a fusion partner of as smallest size as possible without compensating for the expression of the fusion protein. The effectiveness of cleaving target protein off from its fusion partner is

one of the most important factors that determine the economy of a recombinant protein process. For the recovery of target proteins such as PTH whose integrity of the amino-terminal amino acid sequence is crucial for activity, use of enzymatic cleavage method over the chemical method is essential because of the more pronounced specificity of the former than the latter method. For this purpose, several proteolytic enzymes such as factor X_a, thrombin, and enterokinase have been developed and commercially available (116). Limited availability of these enzymes, however, is restricting their application in the development of a commercial-scale process for the production of a recombinant protein using the fusion expression strategy. We solved this problem by taking advantage of a proteolytic enzyme, urokinase, for which commercial-scale production process has already been established in one of our affiliates. Substrate specificity of urokinase has been determined using chromogenic peptide substrates (117). Based on these results, we introduced by site-directed mutagenesis several different amino acid residues in the P3 subsite of a fusion protein substrate and determined the cleavage sequence for urokinase. Culture of *E. coli* harboring the expression plasmid for PTH fusion protein was optimized in terms of the amount of cell mass and the level of expression. Such parameters as media composition, mode of media feed, time and duration of induction, and total culture period were included in the consideration. The PTH fusion protein isolated as inclusion body from the recombinant *E. coli* was solubilized in alkaline solution and then refolded by lowering pH of the solution. Condition for the cleavage of PTH from the fusion protein was optimized in terms of ratio of substrate to protease, reaction temperature and period, and buffer composition. PTH separated from the fusion protein was further purified by using ion exchange column chromatography and C_{18} reversed phase column chromatography. Purity and identity of the purified PTH were confirmed by reversed phase and gel filtration HPLC, SDS-PAGE, mass spectrometry, amino acid composition analysis, and amino-terminal amino acid sequencing. The *in vitro* bioactivity of the purified recombinant hPTH(1-84) comparable to that of synthetic hPTH(1-84) was demonstrated by cAMP stimulation and competitive receptor binding assays using a rat osteosarcoma cell line, UMR 106, which possesses receptors for PTH (118). *In vivo* efficacy of the recombinant hPTH(1-84) to stimulate bone formation was verified in an animal study using rats with osteopenia induced by ovariectomy. Female Sprague-Dawley rats, 90 days old, were either sham operated or ovariectomized and then randomly divided into 5 groups of 10 animals each. Groups 1 and 3 were sham operated, whereas the remaining groups were ovariectomized. Groups 1 and 2 were killed on day 40 post-operation. Starting from day 40, Groups 3, 4, and 5 were administered subcutaneously with vehicle, 10 μg/kg body weight (BW)/day of 17β-estradiol, and 200 μg/kg BW/day of recombinant hPTH(1-84), respectively, 5 days/week for 4 weeks. Histomorphometric analysis of femurs isolated from each group confirmed that the daily injection of recombinant hPTH(1-84) into osteopenic ovariectomized rats induced large improvements in such parameters as trabecular bone volume, trabecular plate thickness, trabecular plate separation, osteoid seam width, and bone appositional rate, all better than the vehicle- or estrogen-treated groups.

REFERENCES

1. Christiansen, C. (1993) *J. Bone Miner. Res.* **8** (Suppl. 2), S475-S480
2. Horowitz, M.C. (1993) *Science* **260**, 626-627
3. Scrip Reports. Osteoporosis : Research, markets, trends and opportunities (1994), PJB Publications Ltd.
4. Urist, M.R., DeLange, R.J., and Finerman, G.A.M. (1983) *Science* **220**, 680-686
5. Aubin, J.E., Turksen, K., and Heersche, J.N.M. (1993), In : M. Noda (ed). *Cellular and Molecular Biology of Bone*, Academic Press, Inc., pp1-45
6. Martin, T.J., Findlay, D.M., *et al.* (1993), In : G.R. Mundy and T.J. Martin (eds). *Physiology and Pharmacology of Bone*, Springer-Verlag Berlin Heidelberg, pp149-183
7. Stein, G.S., Lian, J.B., *et al.* (1989) *Connect. Tissue Res.* **20**, 3-13
8. Stein, G.S., and Lian, J.B. (1993) *Endocrine Rev.* **14**, 424-442
9. Thomson, B.M., Saklatvala, J., and Chambers, T.J. (1986) *J. Exp. Med.* **164**, 104-112
10. Baylink, D.J., Finkelman, R.D., Mohan, S.(1993) *J. Bone Miner. Res.* **8**(Suppl.2), S565-S572
11. Mohan, S., and Baylink, D.J. (1991) *Clin. Orthop.* **263**, 30-48
12. Franchimont, M., and Canalis, E. (1995) *Endocrinol.* **136**, 5469-5475
13. Kawaguchi, H., Pilbeam, C.C., *et al.* (1995) *J. Clin. Invest.* **96**, 923-930
14. Rosen, C.J., and Donahue, L.R. (1995) *Trends Endocrinol. Metabol.* **6**, 235-241
15. Centrella, M., Horowitz, M.C., *et al.* (1994) *Endocrine Rev.* **15**, 27-39
16. Centrella, M., Casinghino, S., and McCarthy, T.L. (1994) *Endocrinol.* **135**, 1611-1620

17. Catherwood, B.D. (1985) *J. Biol. Chem.* **260**, 736-743
18. Suda, T., Takahashi, N., and Martin, T.J. (1992) *Endocrine Rev.* **13**, 66-80
19. Udagawa, N., Takahashi, N., *et al.* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 7260-7264
20. Yoshida, H., Hayashi, S.-I., *et al.* (1990) *Nature* **345**, 442-444
21. Wiktor-Jedrzejczak, W., Bartocci, A., *et al.*(1990) *Proc. Natl. Acad. Sci. USA* **87**, 4828-4832
22. Kodama, H., Yamasaki, *et al.* (1991) *J. Exp. Med.* **173**, 269-272
23. Wiktor-Jedrzejczak, W. (1993) *Leukemia* **7** (Suppl. 2), S117-S121
24. Morohashi, T., Carboz, V.A., *et al.* (1994) *J. Bone Miner. Res.* **9**, 401-407
25. Felix, R., Hofstetter, W., *et al.* (1994) *J. Cell. Biochem.* **55**, 340-349
26. Soriano, P., Montgomery, C., Geske, R., and Bradley, A. (1991) *Cell* **64**, 693-702
27. Boyce, B.F., Yoneda, T., *et al.* (1992) *J. Clin. Invest.* **90**, 1622-1627
28. Lowe, C., Yoneda, T., *et al.* (1993) *Proc. Natl. Acad. Sci. USA* **90**, 4485-4489
29. Quinn, J.M.W., McGee, J.O'D., and Athanasou, N.A. (1993) *Endocrinol.* **134**, 2416-2423
30. Takahashi, S., Goldring, S., *et al.* (1995) *J. Clin. Invest.* **95**, 167-171
31. Uy, H.L., Guise, T.A., *et al.* (1995) *Endocrinol.* **136**, 3207-3212
32. Takahashi, N., Akatsu, T., *et al.* (1988) *Endocrinol.* **123**, 1504-1510
33. Takahashi, N., Yamada, H., *et al.* (1988) *Endocrinol.* **122**,1373-1382
34. Lee, S.K., Goldring, S.R., and Lorenzo, J.A. (1995) *Endocrinol.* **136**, 4572-4581
35. Baron, R., Chakraborty, M., *et al.* (1993). In : G.R. Mundy and T.J. Martin (eds). *Physiology and Pharmacology of Bone*, Springer-Verlag Berlin Heidelberg, pp111-147
36. Ross, F.P., Chappel, J., *et al.* (1993) *J. Biol. Chem.* **268**, 9901-9907
37. Davies, J., Warwick, J., *et al.* (1989) *J. Cell Biol.* **109**, 1817-1826
38. Aarden E.M., Burger, E.H., and Nijweide, P.J. (1994) *J. Cell. Biocehm.* **55**, 287-299
39. Klein-Nulend, J., van der Plas, A., *et al.* (1995) *FASEB J.* **9**, 441-445
40. Miller, S.C., and Jee, W.S.S. 91987) *Calcif. Tissue Int.* **41**, 1-5
41. Weryha, G., and Leclère, J. 91995) *Horm. Res.* **43**, 69-75
42. Raisz, L.G. (1993) *J. Bone Miner. Res.* **8** (Suppl. 2), S457-S465
43. Raisz, L.G., and Shoukri, K.C. (1993). In : G.R. Mundy and T.J. Martin (eds). *Physiology and Pharmacology of Bone*, Springer-Verlag Berlin Heidelberg, pp299-331
44. Manolagas, S.C., and Jilka, R.L. (1995) *N. Engl. J. Med.* **332**, 305-311
45. Turner, R.T., Riggs, B.L., and Spelsberg, T.C. (1994) *Endocrine Rev.* **15**, 275-300
46. Prince, R.L. (1994) *Endocrine Rev.* **15**, 301-309
47. Jilka, R.L., Hangoc, G., *et al.* (1992) *Science* **257**, 88-91
48. Poli, V., Balena, R., *et al.* (1994) *EMBO J.* **13**, 1189-1196
49. Matsushita, M., Tsuboyama, T., and Kasai, R. (1986) *J. Am. Pathol.* **125**, 276-283
50. Nicolas, V., Prewett, A., and Bettica, P. (1994) *J. Clin. Endocrinol. Metab.* **78**, 1011-1016
51. Marcus, R., Butterfiels, G., *et al.* (1990) *J. Clin. Endocrinol. Metab.* **70**, 519-527
52. Rudman, D., Feller, A.G., *et al.* (1990) *N. Engl. J. Med.* **323**, 1-6
53. Lindsay, R., Hart, D.M., and Clark, D.M. (1984) *Obstet. Gynecol.* **63**, 759-763
54. Davidson, N.E. (1992) *N. Engl. J. Med.* **326**, 885-886
55. Ke, H.Z., Simmons, H.A., *et al.* (1995) *Endocrinol.* **136**, 2435-2441
56. Yang, N.N., and Hardikar, S. (1994) *J. Bone Miner. Res.* **9** (Suppl. 1), S144
57. Austin, L.A., and Heath III, H. (1981) *N. Engl. J. Med.* **304**, 269-278
58. Jung, A., Bisaz, S., and Fleisch, H. (1973) *Calcif. Tissue Res.* **11**, 269-280
59. Hughes, D.E., MacDonald, B.R., *et al.* (1989) *J. Clin. Invest.* **83**, 1930-1935
60. Flanagan, A.M., and Chambers, T.J. (1989) *Bone Miner.* **6**, 33-43
61. Fleisch, H. (1993). In : G.R. Mundy and T.J. Martin (eds). *Physiology and Pharmacology of Bone*, Springer-Verlag Berlin Heidelberg, pp377-418
62. Yoneda, T., Lowe, C., *et al.* (1993) *J. Clin. Invest.* **91**,2791-2795
63. Sato, M., Sardana, M.K., *et al.* (1990) *J. Cell Biol.* **111**, 1713-1723
64. Fisher, J.E., Caulfield, M.P., *et al.* (1993) *Endocrinol.* **132**, 1411-1413
65. Horton, M.A., Dorey, E. L., *et al.* (1993) *J. Bone Miner. Res.* **8**, 239-247
66. Pierce, W.M., Jr., and Waite, L.C. (1987) *Proc. Soc. Exp. Biol. Med.* **186**, 96-102
67. Sundquist, K., Lakkakorpi, P., *et al.* (1990) *Biochem. Biophys. Res. Comm.* **168**, 309-313
68. Hill, P.A., Murphy, G., *et al.* (1994) *J. Cell Sci.* **107**, 3055-3064
69. Farley, J.R., Wergedal, J.E., and Baylink, D.J. (1983) *Science* **222**, 330-332
70. Riggs, B.L., Hodgson, S.A., O'Fallon, W.M., et al. (1990) *N. Engl. J. Med.* **322**, 802-809
71. Wolinsky, I., Simkin, A., and Guggenheim, K. (1972) *Am. J. Physiol.* **223**, 46-50
72. Pak, C.Y.C., Zerwekh, J.E., and Antich, P. (1995) *Trends Endocrinol. Metab.* **6**, 229-234
73. McSheehy P.H.J., and Chambers, T.J. (1986) *Endocrinol.* **118**, 824-828
74. Abou-Samra, A.-B., Jüppner, H., *et al.* (1992) *Proc. Natl. Acad. Sci. USA* **89**, 2732-2736
75. Jüppner, H., Abou-Samra, A.-B., *et al.* (1991) *Science* **254**, 1024-1026
76. Schipani, E., Karga, H., *et al.* (1993) *Endocrinol.* **132**, 2157-2165

77. Schneider, H., Feyen, J.H.M., *et al.* (1993) *Eur. J. Pharmacol.* **246**, 149-155
78. Tregear, G.W., van Rietschoten, J., *et al.* (1973) *Endocrinol.* **93**, 1349-1353
79. Rosenblatt, M. (1981) *Pharmacol. Ann.* **11**, 53-86
80. Fujimori, A., Cheng, S.-L., Avioli, L.V., and Civitelli, R. (1992) *Endocrinol.* **130**, 29-36
81. Goltzmann, D., Peytremann, A., *et al.* (1975) *J. Biol. Chem.* **250**, 3199-3203
82. Donahue, H.J., Fryer, M.J., *et al.* (1988) *J. Biol. Chem.* **263**, 13522-13527
83. Born, W., Loveridge, N., *et al.* (1988) *Endocrinol.* **123**, 1848-1853
84. Tam, C.S., Heersche, J.N.M., *et al.* (1982) *Endocrinol.* **110**, 506-512
85. Gunness-Hey, M., and Hock, J.M. (1984) *Metab. Bone Dis. Rel. Res.* **5**, 177-181
86. Hori, M., Uzawa, T., *et al.* (1988) *Bone Miner.* **3**, 193-199
87. Hock, J.M., Hummert, J.R., *et al.* (1989) *J. Bone Miner. Res.* **4**, 449-458
88. Liu, C.C., Kalu, D.N., *et al.* (1991) *J. Bone Miner. Res.* **6**, 1071-1079
89. Kimmel, D.B., Bozzato, R.P., *et al.* (1993) *Endocrinol.* **132**, 1577-1584
90. Wronski, T.J., and Yen, C.-F. (1994) *Bone* **15**, 51-58
91. Wronski, T.J., Yen, C.-F., Qi, H., and Dann, L.M. (1993) *Endocrinol.* **132**, 823-831
92. Sogaard, C.H., Wronski, T.J., *et al.* (1994) *Endocrinol.* **134**, 650-657
93. Gabrielsen, O.S., Reppe, S., *et al.* (1990) *Gene* **90**, 255-262
94. Hogset, A., Blingsmo, O.R., *et al.* (1990) *J. Biol. Chem.* **265**, 7338-7344
95. Gardella, T.J., Rubin, D., *et al.* (1990) *J. Biol. Chem.* **265**, 15854-15859
96. Harder, M.P.F., Sanders, E.A., *et al.* (1993) *Appl. Microbiol. Biotechnol.* **39**, 329-334
97. Oshika, Y., Yamada, T., *et al.* (1994) *Int. J. Peptide Protein Res.* **43**, 441-447
98. Mosekilde, L., Sogaard, C.H., *et al.* (1991) *Endocrinol.* **129**, 421-428
99. Ejersted, C., Andreassen, T.T., *et al.* (1994) *Eur. J. Endocrinol.* **130**, 201-207
100. Gunness-Hey, M., and Hock, J.M. (1989) *Bone* **10**, 447-452
101. Yamamoto, N., Takahashi, H.E., *et al.* (1993) *Bone MIner.* **23**, 333-342
102. Cheng, P.-T., Chan, C., and Müller, K. (1995) *J. Bone Miner. Res.* **10**, 119-126
103. Shen, V., Dempster, D.W., *et al.* (1993) *J. Clin. Invest.* **91**, 2479-2487
104. Shen, V., Birchman, R., *et al.* (1995) *J. Clin. Invest.* **96**, 2331-2338
105. Mosekilde, L., Danielssen, C.C., and Gasser, J. (1994) *Endocrinol.* **134**, 2126-2134
106. Dempster, D.W., Cosman, F., *et al.* (1993) *Endocrine Rev.* **14**, 690-709
107. Tsai, K.S., Ebeling, P.R., and Riggs, B.L. (1989) *J. Clin. Endocrinol. Metab.* **69**, 1024-1027
108. Hodsman, A.B., and Steer, B.M. (1993) *Bone* **14**, 523-527
109. Reeve, J., Meunier, P.J., *et al.* (1980) *Br. Med. J.* **280**, 1340-1344
110. Slovik, D.M., Rosenthal, D.I., *et al.* (1986) *J. Bone Miner. Res.* **1**, 377-381
111. Hodsman, A.B., Steer, B.M., Fraher, L.J., and Drost, D.J. (1991) *Bone Miner.* **14**, 67-83
112. Finkelstein, J.S., Klibanski, A., *et al.* (1994) *N. Engl. J. Med.* **331**, 1618-1623
113. Canalis, E., Centrella, M., Burch, W., and McCarthy, T.L. (1989) *J. Clin. Invest.* **83**, 60-65
114. Watson, P., Lazowski, D., *et al.* (1995) *Bone* **16**, 357-365
115. Rixon, R.H., Whitfield, J.F., *et al.* (1994) *J. Bone Miner. Res.* **9**, 1179-1189
116. Marston, F.A.O. (1986) *Biochem. J.* **240**, 1-12
117. Lottenberg, R., Christensen, U., *et al.* (1981) *Methods Enzymol.* **80**, 341-361
118. Partridge, N.C., Alcorn, D., *et al.* (1983) *Cancer Res.* **43**, 4308-4314