Mechanisms Involved in Skeletal Anabolic Therapies

T.J. MARTIN,[a] J.M.W. QUINN,[a] M.T. GILLESPIE,[a] K.W. NG,[a] M.A. KARSDAL,[c] AND N.A. SIMS,[a,b]

[a]*St. Vincent's Institute of Medical Research, Fitzroy Victoria 3065, Australia*

[b]*Department of Medicine, University of Melbourne, St. Vincent's Hospital, Fitzroy Victoria 3063, Australia*

[c]*Nordic Bioscience A/S, Herlevhovedgade 207, DK-2730 Herlev, Denmark*

ABSTRACT: Since parathyroid hormone (PTH) is the only proven anabolic therapy for bone, it becomes the benchmark by which new treatments will be evaluated. The anabolic effect of PTH is dependent upon intermittent administration, but when an elevated PTH level is maintained even for a few hours it initiates processes leading to new osteoclast formation, and the consequent resorption overrides the effects of activating genes that direct bone formation. Identification of PTH-related protein (PTHrP) production by cells early in the osteoblast lineage, and its action through the PTH1R upon more mature osteoblastic cells, together with the observation that PTHrP± mice are osteoporotic, all raise the possibility that PTHrP is a crucial paracrine regulator of bone formation. The finding that concurrent treatment with bisphosphonates impairs the anabolic response to PTH, adds to other clues that osteoclast activity is necessary to complement the direct effect that PTH has in promoting differentiation of committed osteoblast precursors. This might involve the generation of a coupling factor from osteoclasts that are transiently activated by receptor activator of nuclear factor-κB ligand (RANKL) in response to PTH.

New approaches to anabolic therapies may come from the discovery that an activating mutation in the LRP5 gene is responsible for an inherited high bone mass syndrome, and the fact that this can be recapitulated in transgenic mice, whereas inactivating mutations result in severe bone loss. This has focused attention on the Wnt/frizzled/β-catenin pathway as being important in bone formation, and proof of the concept has been obtained in experimental models.

KEYWORDS: osteoblast; osteoclast; parathyroid hormone; Wnt signaling

Address for correspondence: T. John Martin, St. Vincent's Institute of Medical Research, 9 Princes Street, Fitzroy Vic 3065, Australia. Voice: 61-3-9288-2480; fax: 61-3-9416-2676.
e-mail: jmartin@svi.edu.au

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FORMATION OF BONE

In the adult human skeleton, approximately 5–10% of the existing bone is replaced every year through the process of bone remodeling. The remodeling process, which continues throughout adult life, is an integral part of the calcium homeostatic system and provides a mechanism for self-repair and adaptation to physical stress. The cellular sequence is always initiated by osteoclastic bone resorption to be followed by osteoblastic new bone formation. This sequence of events is initiated asynchronously throughout the skeleton, at sites that are geographically and chronologically separated from each other. Both bone formation and resorption occur at the same place so that there is no change in the shape of the bone.

The maintenance of a normal, healthy skeletal mass depends on information transfer taking place among osteoblasts, osteoclasts, immune cells, and constituents of the bone matrix. Remodeling thus maintains the mechanical integrity of the skeleton by replacing old bone with new bone.[1–5] The maintenance of adequate trabecular and cortical bone requires that bone formation and resorption should be balanced, such that a high or low level of resorption is usually associated with a similar change in the level of bone formation. The theory that resorption is followed by an equal amount of formation has come to be known as "coupling." However, during life, the effects of growth and aging, including changes in mechanical stress mean that this theory of equal bone replacement rarely holds true. During growth there is a positive balance, with the amount of bone replaced at individual basic multicellular units (BMUs) exceeding that lost,[3] and with aging there is a negative balance at individual BMUs,[6] with gradual attrition of bone. In common metabolic states, such as postmenopausal osteoporosis, while coupling exists and both bone formation and resorption are occurring at a higher level than normal, the amount of bone formed is not equal to that resorbed and bone density is therefore reduced.

Until the early 1980s it was understood that bone metabolism was regulated by circulating hormones. The discoveries in subsequent years revealed that, although circulating hormones are important controlling factors, the key influences are locally generated cytokines that influence bone cell function and communication in complex ways, and often are themselves regulated by the hormones. Any approach to treatment of bone disease requires an understanding of these relationships. Until recently the established means of osteoporosis prevention and treatment have been limited entirely to drugs that inhibit bone resorption. Now that the first bone-forming treatment has been developed, and others are being investigated as new prospects, it is essential that the local communication networks in bone be taken into consideration. The processes central to successful anabolic skeletal therapies are those that enhance osteoblast differentiation, most importantly doing so in ways that preserve normal bone shape and structure. Thus, the integrity of the processes involved in the normal control of bone turnover needs to be maintained in the course of any anabolic

treatment. This article focuses on the cellular and molecular mechanisms that are relevant to current and future approaches to anabolic therapies.

TRANSCRIPTIONAL CONTROLS

Cbfa1 (Runx2) is an essential and early transcriptional regulator of osteoblast differentiation.[7] It also controls bone cell function by maintaining the differentiated phenotype of the osteoblast in maturity. Transgenic overexpression of a dominant negative form of runx2 postnatally in mice led to decreased production of runx2, as well as diminished expression of the genes required for the differentiated osteoblast.[8] Runx2 is central to the replenishment of osteoblasts after bone loss, a key requirement in restoring bone.

The search for transcription factors other than runx2 led to the identification of a novel zinc finger containing transcription factor, osterix (Osx), which is specifically expressed in developing bones and not in other tissues. Mice rendered null for the *osx* gene did not develop mineralized bone, but, like the *runx2-/-* mice, had an entirely cartilaginous skeleton and died at birth.[9] In *Osx-/-* mice, runx2 mRNA was expressed at the same level as in the wild type, but the mutant mice had no Osx mRNA. This suggests that Osx is an important transcription factor in osteoblast differentiation, which functions downstream from runx2 and perhaps acts cooperatively with it. The bone morphogenetic proteins (BMPs) are important in osteoblast differentiation through their paracrine role in bringing into play signaling mechanisms that are crucial for the process,[10] including sonic and indian hedgehog, and Wingless-type (Wnt) signaling.

However important runx2 is in osteoblast differentiation and bone formation, it is clearly subject to regulatory mechanisms mediated by other transcription factors, growth factors, and hormones.[7] These include parathyroid hormone (PTH), which activates the *runx2* promoter, increasing production of mRNA and protein for runx2 in osteoblasts, raising the question of whether this might contribute to the anabolic action of PTH.[11] This regulatory pathway contains a series of molecular steps that very likely can serve as targets for the development of drugs to influence bone formation.

THE WNT SIGNALING PATHWAY AND BONE FORMATION

Intriguing insights into the control of bone mass come from discoveries of mutations in a gene associated with the osteoporosis pseudoglioma syndrome, and with a high bone mass syndrome. The genetic abnormalities consist of inactivating mutations in the gene for low-density lipoprotein receptor-related protein 5 (LRP5), resulting in impaired bone mass and severe osteoporosis, with heterozygous carriers also having reduced bone mass and an increased

incidence of osteoporotic fractures.[12] On the other hand, an activating mutation of the same gene in another kindred is associated with greatly increased mass of bone that is nevertheless of normal shape and architecture.[13,14] *In vitro* studies showed that the normal inhibition of Wnt signaling by another protein, the Wnt antagonist, Dickkopf (Dkk 1) was defective in the presence of the mutant LRP5, providing a molecular explanation for the increased activity of the Wnt signaling pathway.

LRP5 is a single-pass membrane receptor that forms part of a complex necessary for activation in the Wnt signaling pathway. LRP5 interacts with the Wnt-frizzled ligand–receptor complex, resulting in the inhibition of β-catenin phosphorylation by glycogen synthase kinase-3β (GSK-3β). Since GSK-3β activity facilitates ubiquitin-mediated breakdown of β-catenin, the LRP5 effect is to prevent this, allowing accumulation of β-catenin and hence its translocation to the nucleus, where it interacts with TCF/LEF transcription factors to activate gene transcription. LRP5 and LRP6 are co-receptors for Wnt proteins, with LRP5 inhibited by the Wnt antagonists, Dkk-1 or Dkk-2, and activated by Dkk-3 or Dkk-4. A potential link between the PTH and Wnt signaling pathways arises from *in vivo* gene array studies in rats treated with PTH, in which several components of the Wnt–receptor complex were upregulated in PTH-treated rat bone, and furthermore, PTH treatment of osteoblastic cells *in vitro* activated the TCF/LEF transcriptional pathway.[15] Notably, activating the Wnt signaling pathway in rats by treatment with a GSK-3β inhibitor resulted in increased bone formation.[16]

These findings together have revealed a previously unrecognized control pathway for osteoblast differentiation and function and bone growth, a pathway studied extensively in the development in many organisms, as well as in cancer. The control mechanisms for this pathway are complex, providing several points that are the focus of attention toward the development of drugs anabolic for bone. Its very complexity, the number of participants, and the likely involvement of this pathway in cell proliferation control and in neoplastic processes in some tissues, provide major challenges.

STIMULATION OF BONE FORMATION BY PTH

The only means currently available for skeletal anabolic therapy is intermittent treatment with PTH(1-34), approved in the United States as "Forteo" for the treatment of osteoporosis.[17] Other active forms of PTH, including PTH(1-84) are under study, including being in clinical trial. The efficacy of PTH in promoting bone formation is such that it sets a standard for any new treatments that are to be developed. It is therefore appropriate to consider its actions in further detail.

The ability of intermittent injections of PTH to increase bone formation was already recognized in the 1930s. Recent clinical studies established that

PTH(1-34) has a powerful anabolic effect, revealing it as the first therapy capable of restoring lost bone.[17] Not surprisingly, PTH-related protein (PTHrP) exerts similar actions, with PTHrP (1-34), (1-36), and (1-74) having all been shown to be anabolic in rodents.[18,19] Clinical data from the measurement of bone markers indicate that PTHrP(1-36) is also anabolic in human subjects.[20]

Growth Factors as Mediators of PTH Anabolic Response

The two general mechanisms proposed for the PTH anabolic effect are promotion by PTH of differentiation of committed osteoblast precursors,[21] and inhibition of osteoblast apoptosis by PTH.[22] PTH administered intermittently *in vivo* enhances the production of proteins associated with bone formation, including runx2, osteocalcin, and type I collagen. There is also evidence from a number of approaches that the PTH anabolic response could be mediated at least in part, through increased production and/or activation of growth factors known to be capable of enhancing bone formation.[23] Insulin-like growth factor-1 (IGF-1) and transforming growth factor (TGF)-β are two of the major growth factors of bone. IGF-1 is provided in a latent form in matrix because of the regulated production of several specific binding proteins that sequester it.[24] The bone matrix stores large amounts of latent TGF-β, which can be activated through the acid conditions of bone resorption[25] or by proteases produced by osteoblasts.[26] PTH treatment of osteoblasts has been shown to activate TGF-β[26] as well as to enhance IGF-1 production by osteoblasts and by bone in organ culture. Such mechanisms could contribute to the PTH anabolic effect. Consistent with this possibility is the finding that the anabolic response to PTH is lost in mice rendered null for *IGF-1*.[27]

Anabolic Effect of PTH Requires Transient Exposure to Increased Hormone

The ability of PTH to promote bone formation is dependent upon the hormone being administered intermittently in a way that yields a peak blood level that is not maintained.[17,28] In that circumstance, processes are initiated in bone, which result in anabolic effects, presumably as a result of the activation of genes responding specifically to a transiently activated signaling system that requires a rapid increase in PTH or PTHrP, with a rapid decline to preexisting levels. On the other hand, if PTH or PTHrP is infused, or administered in such a way that elevated plasma levels are maintained, the dominant effect is stimulation of osteoclast formation and bone resorption, to the extent that these override any anabolic response. Studies in the rat *in vivo* support this view. Infusion of PTH into rats caused a robust and sustained increase in RANKL and decrease in osteoprotegerin (OPG) production in bone, which preceded hypercalcemia

and enhanced osteoclast formation. In these conditions also, sustained elevated levels of PTH resulted in decreased expression of genes associated with bone formation.[29] These included cbfa1, osteocalcin, bone sialoprotein, and type 1 collagen. In contrast, repeated single injections of PTH triggered a rapid but transient increase in the RANKL/OPG ratio, with a peak 1 h after PTH injection and a rapid return to control levels. This was associated with increased bone formation and enhanced expression of the genes associated with bone formation.[30] Finally, and most importantly, Holtrop *et al*.[31] showed that intravenous injection of PTH in young rats resulted in transient activation of osteoclasts *in vivo*, evident within 30 min, and followed only some hours later at high PTH doses by increased osteoclast number. In light of current knowledge, it is most likely that rapid, transient activation of osteoclasts following PTH injection is the result of rapid induction of RANKL expression in cells of the osteoblast lineage.

The results of these experiments raised the question: "Is an effect of PTH upon osteoclasts necessary for the anabolic effect?" A number of lines of evidence are consistent with this possibility.

Osteoclast Activity and Bone Formation Responses

When sheep were treated with PTH for 3 months the anabolic effect was significantly reduced when a bisphosphonate (tiludronate) was co-administered.[32] In ovariectomized rats treated with PTH, bone formation parameters (assessed by histomorphometry) were reduced when co-treatment was provided either with estrogen or with the bisphosphonate, risedronate.[33] A similar conclusion, that osteoclast activity is necessary for the anabolic response to PTH, came from studies in mice rendered null for the *c-fos* gene, which are osteopetrotic because they cannot develop osteoclasts, and furthermore fail to show an anabolic response to PTH.[34] This evidence from animals is supported by recent clinical studies. Treatment of patients with osteoporosis concomitantly with PTH and a bisphosphonate resulted in significant early blunting of the anabolic response to PTH,[35,36] with the obvious implication that combining the anabolic PTH with antiresorptive bisphosphonate would be contraindicated.[35–38]

Observations made in both human and mouse genetics suggest that the osteoclast could be a source of an activity that contributes to bone formation. In individuals with the osteopetrotic syndrome, ADOII, due to inactivating mutations in the chloride-7 channel (ClC-7), bone resorption is deficient because of the failure of the osteoclast acidification process. These patients have increased osteoclast numbers, but bone formation is nevertheless normal, rather than diminished as might be expected because of the greatly impaired resorption.[39] Furthermore, in mice deficient in either c-*src,*[40] ClC-7,[41] or tyrosine phosphatase epsilon,[42] bone resorption is inhibited without inhibition of formation. In these three knockout mouse lines osteoclast resorption is greatly reduced by the mutation, although osteoclast numbers are not reduced. Indeed,

osteoclast numbers are actually increased in vacuolar ATPase (V-ATPase)-deficient mice because of reduced osteoclast apoptosis. A possibility is that these osteoclasts, although unable to resorb bone, are nevertheless capable of generating a factor (or factors) required for bone formation. An illustration of this concept is provided in studies of reparative bone growth around calcium phosphate implants, in which inhibition of bone resorption by the V-ATPase inhibitor, bafilomycin, led to increased nonresorbing osteoclasts surrounding the neo-implants, resulting in a large increase in bone formation.[43] Thus the bone formation increase was not dependent on resorption by the osteoclasts.

The cytokines that signal through glycoprotein (gp)130 play an important role in intercellular communication processes in bone, with evidence indicating that they can be involved in the regulation of both bone resorption and formation.[44–46] We addressed this by studying mice in which each of the two gp130-dependent signaling pathways was specifically attenuated, and found that inactivation of the SHP2/ras/MAPK signaling pathway yielded mice with greater osteoclast numbers and bone resorption, as well as greater bone formation than wild-type mice. The net effect was a decreased amount of bone in these mice. When these mice were crossed with IL-6 null mice, the resulting mutants had similarly high osteoclast numbers and increased bone resorption, but without any increase in bone formation and therefore with extremely low bone mass. Thus resorption alone is insufficient to promote the coupled bone formation, but the active osteoclasts are the likely source of an activity that is IL-6 dependent, but not necessarily IL-6 itself.[46,47]

Our interpretation of the foregoing and other data is that what is needed for the full expression of the anabolic action of PTH, in addition to its direct effects on committed preosteoblasts, is a transient effect on the osteoclast, achieved by promoting *activation,* rather than *formation,* of osteoclasts.[47] This is an important distinction, and the precise way in which the osteoclast is involved in the anabolic process needs to be clearly understood because of the implications for sequential or combined use of therapeutic resorption inhibitors and anabolic agents, and for the development of new anabolic agents. FIGURE 1 illustrates this schematically, indicating the known effect of PTH to increase osteoclast activity indirectly by acting on osteoblastic cells to increase RANKL. The RANKL-activated osteoclasts are depicted as releasing a biological activity that enhances osteoblast maturation, either independently or cooperatively with PTH, or with the locally important factors promoting bone formation. The impaired anabolic response to PTH when there is a complete blockade of osteoclasts, either through their impaired formation, as in *c-fos* deficient mice, or resulting from prolonged bisphosphonate treatment, implies that the full expression of the anabolic effect might require a contribution from the active osteoclast. The genetic data implies further that inhibition of osteoclastic resorption, e.g., by preventing acidification, might nevertheless provide osteoclasts that are capable of producing this anabolic stimulus. An interesting further implication is that resorption inhibitors based

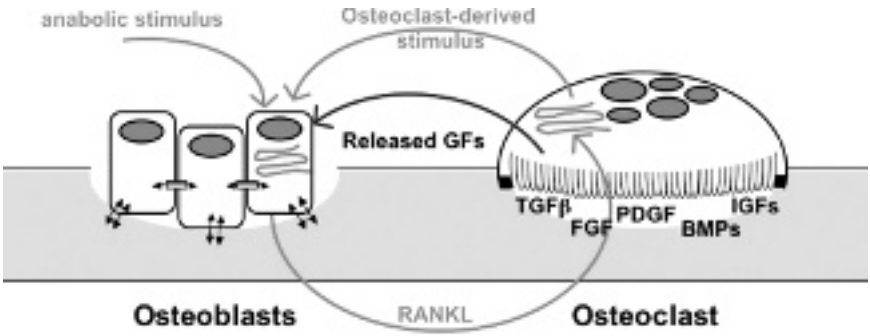

FIGURE 1. Osteoclasts can contribute to bone formation either by their promotion of resorption, with release of stored growth factors from matrix, or by the release from transiently activated osteoclasts of activity that contributes to anabolic effects on bone. (Modified from Martin, T.J. & N.A.Sims[47] with permission).

on inhibition of acidification of *c-Src* might not impair the anabolic response to PTH.

Significance of PTHrP Production in Bone

The discovery of PTHrP production in bone[48,49] raised the intriguing possibility that it has important local actions in bone, not only those reflecting the anabolic action of PTH, but also possibly producing other effects through actions of differently processed forms of the molecule. The cells producing PTHrP in bone are likely distinct from those responding, at least through the PTH1R. In isolated cells from rat calvariae, for example, cells of early enzyme digests were those most abundantly producing PTHrP, whereas PTH responses through the common PTH/PTHrP receptor (PTH1R) were enriched in the osteoblast-rich populations of later digests (TABLE 1). Furthermore, in studying differentiation of mouse stromal osteoblast precursors, decline in PTHrP mRNA production was associated with increase in PTH1R receptor mRNA and PTH- induced cAMP response.[50]

One function that has been studied extensively is the role of PTHrP in endochondral bone formation. Targeted disruption of the genes for PTHrP or PTH1R in mice resulted in death in the perinatal period with gross skeletal abnormalities consistent with chondrodysplasia.[51,52] Histological studies suggest a central role of PTHrP in fetal endochondral bone formation through its actions in maintaining a pool of proliferating chondrocytes, inhibition of terminal chondrocyte differentiation, retardation of cartilage matrix mineralization, and differentiation of periosteal mesenchymal precursors into cells of the chondrocytic or osteoblastic lineages.

It appears that PTHrP might be involved in intramembranous bone formation also. In an experimental model of this process in the rabbit bone, formation

TABLE 1. Alkaline Phosphatase Activity, PTHrP Production and cAMP Response in Sequentially Digested Rat Calvarial Cells

	Alk Phos (nmol/min/mg protein)	PTHrP (fmol/10^6 cells)	cAMP (response to 10 nM PTHrP)
1	32 ± 1.9	20.9 ± 2.1	0.16
2	43 ± 4.1	9.8 ± 0.5	0
3	63 ± 7.0	3.9 ± 0.1	0.11
4	214 ± 15	6.7 ± 0.2	7.5
5	291 ± 16	7.5 ± 0.3	6.5
6	714 ± 24	4.2 ± 0.1	23

cAMP data are expressed as the fold response, with triplicate assays for control and treatment with 10 nM PTHrP. (Used with permission from Suda *et al.*[48])

begins with transformation of primitive marrow mesenchymal cells into trabecular bone without any cartilage intermediate, and the synchronized appearance of hemopoietic marrow elements follows the onset of matrix mineralization. In this model, cells of the osteoblast lineage consistently expressed PTHrP mRNA and protein throughout the bone formation sequence, with prominent production by cuboidal, actively synthesizing osteoblasts, and weaker expression in lining cells on the mineralized trabeculae.[49] These observations, together with those of Suda *et al.*,[48] are consistent with a role for PTHrP in the differentiation of mesenchymal precursors to the osteogenic lineage.

A most important observation made in the course of the genetic experiments was that heterozygous *PTHrP* null mice were shown to be phenotypically normal at birth, but by 3 months of age they demonstrated a reduction in trabecular bone volume, and an increase in adipocytes was observed in the bone marrow.[53] The absolute requirement of PTHrP for normal bone integrity not only in the fetus but also postnatally, together with the finding that PTHrP haploinsufficiency is associated with less bone and with the preferential differentiation of stromal mesenchymal cells into adipocytes rather than osteocytes, all point to PTHrP as a critical molecule in the development and maintenance of bone. Evidence for a crucial role of locally generated PTHrP in bone remodeling was published while this article was in press. Miao *et al.* established this in mice in which *PTHrP* gene was specifically ablated in osteoblasts.[54]

What are the ways in which PTHrP can act as a paracrine/autocrine factor in bone? The pharmacologic effects of intermittent versus sustained PTH/PTHrP treatment are striking and very different. If the behavior of osteoblasts in response to stimulation through PTHR1 requires this type of variation in the delivery of the relevant ligand, can PTH, as a circulating peptide hormone, achieve this? That is doubtful. On the other hand, the regulated, local production of PTHrP could fulfill this role, with its regulation being the result of hormonal, cytokine, or neural control. In the case of PTHrP there is an added possibility that biological activities within the remainder of the molecule could influence

local events, either through independent processes or by modifying actions through the PTH1R.

In summary, the efficacy of PTH in promoting bone formation provides a benchmark for the evaluation of new anabolic therapies. New approaches to anabolic treatment might also be expected from increased understanding of the molecular and cellular basis of this action of PTH/PTHrP, and by answering the question whether the primary physiological regulator of bone formation is the paracrine factor, PTHrP, rather than the hormone, PTH. Other pathways also offer targets for the development of skeletal anabolics, with the pathway of most current interest the Wnt signaling pathway, which may even interact with PTH/PTHrP.

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