Expert Opinion

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Parathyroid hormone and leptin – new peptides, expanding clinical prospects

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There are three injectable and one oral bone-building (i.e., bone anabolic) parathyroid hormone (PTH) peptides. One of the four, Lilly's injectable teriparatide (Forteo™), is currently being used, and the other three are in clinical trials. They are being used or assessed only for treating postmenopausal osteoporosis. However, their potential clinical targets now extend far beyond osteoporosis. They can accelerate the mending of even severe non-union fractures; they will probably be used to strengthen the anchorage of prostheses to bone; they have been shown to treat psoriasis that has resisted other treatments; they can increase the size of haematopoietic stem cell proliferation and accelerate the endogenous repopulation or repopulation by donor transplants of bone marrow depleted by chemotherapeutic drugs; and they may prevent vascular ossification. Leptin, a member of the cytokine superfamily has a PTH-like osteogenic activity and may even partly mediate PTH action. But leptin has two drawbacks that cloud its therapeutic future. First, apart from directly stimulating osteoblastic cells, it targets cells in the hypothalamic ventromedial nuclei and through them it reduces oestrogenic activity by promoting osteoblast-suppressing adrenergic activity. Second, it stimulates vascular and heart valve ossification, which leads to such events as heart failure and diabetic limb amputations.

Keywords: atherosclerosis, bone marrow, diabetes, fractures, hypothalamus, keratinocytes, leptin, orthopaedic implants, osteoporosis, psoriasis, PTH, PTHrP, PTHr1 receptor, vascular ossification

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1. Introduction

In the new millennium, humans will be travelling to Mars and perhaps beyond with a vital, but inappropriately designed, piece of equipment: a skeleton that responds to microgravity by self-destructing. Meanwhile, back on Earth, growing numbers of ageing men and many more women are suffering from crippling bone loss and need to replace degenerating joints.

During the first decade after menopause, all women suffer an accelerating loss of bone, which in some is severe enough to result in spontaneous crushing of vertebrae by normal spinal bending and the breaking of other bones by the often large forces put on them during normal muscle pulling. This is osteoporosis, which all too often requires prolonged and expensive convalescent care, and leads to further bone loss caused by immobilisation, the incapacitation and mental stress of which may even kill the person. The ominously accelerating micro-architectural deterioration and fragility of postmenopausal women's bones are caused by an oestrogen shortage. The slower development of osteoporosis in ageing men is also partly due to a dwindling supply of oestrogen made by bone cells from circulating testosterone and is needed for bone maintenance just as it is in women.

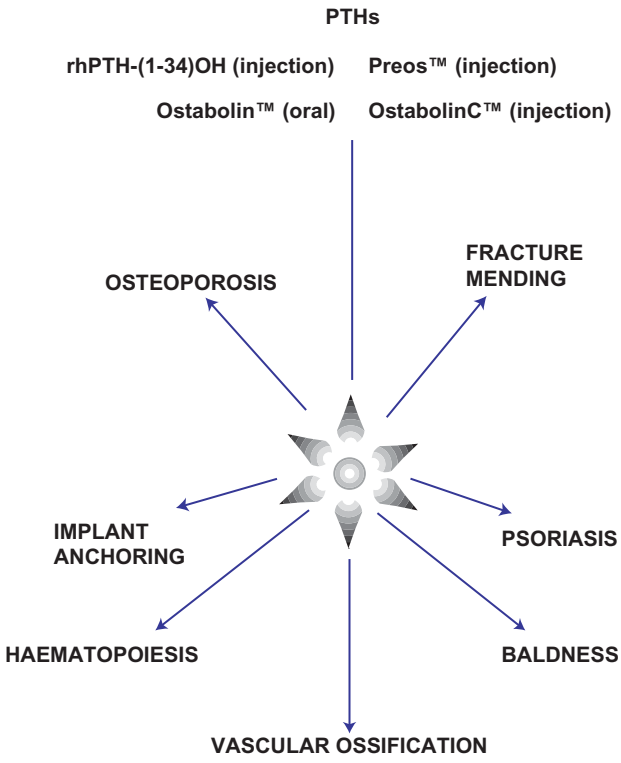

Figure 1. The many emerging clinical uses for the four PTH peptides that are currently either in clinical use or in various stages of clinical trial for treating postmenopausal osteoporosis.

PTH: Parathyroid hormone; rh: Recombinant human.

The oestrogen drop triggers a vicious cycle of a disproportionally rising resorptive arm of the coupled resorption–microcrack-repairing remodelling refilling mechanism and a consequently rising fragility and increasing microfracturing [1-4]. This vicious cycle is due to increasing generation, longevity and activity of the microfracture-excavating osteoclasts in the growing numbers of crack-repairing osteoclast–osteoblast teams in the increasingly microcrack-prone bones. The accelerating generation of hyperactive osteoclasts can be stopped or at least significantly reduced by oestrogens and selective oestrogen receptor modulators (SERMs), as well as by osteoclast-killing bisphosphonates and calcitonin [1-4]. It can also be stopped in its tracks by injecting the osteoblastic cells' osteoclastogenesis-restraining protein, osteoprotegerin [1-4]. But all of these agents harden bone by prolonging mineralisation, and they indirectly cause a limited amount of bone growth because the crippling and killing of osteoclasts enables the unaffected osteoblasts to fill in the existing holes without new ones being dug at the same time [1-4]. However, they do not stimulate osteoblasts to make bone; they are resorption arrestors, antiresorptives and not bone anabolic agents [1-4]. What the growing number of seniors with weakening bones need are bone anabolic drugs that directly stimulate bone growth independently of suppressing osteoclasts and actually rebuild their deteriorating bone microstructure.

Here we will introduce two kinds of osteogenic peptide; the parathyroid hormones (PTHs) and the cytokine leptin. It was claimed in 2000 that leptin is an anti-anabolic operating through the hypothalamus [5] but, as shall be shown, there is now solid evidence for it being a bone anabolic, albeit one with problems. However, the spotlight must first be put on the leading anabolic agents, the potent 84 amino acid PTH and some of its 31 and 34 amino acid fragments, which are in clinical trial, or, as in the case of Lilly's Forteo™ (recombinant human [rh]PTH-[1-34]OH), now actually being used to treat osteoporosis [1-4]. Although the PTHs are known today only as potent osteogenic drugs for treating osteoporosis, they will soon be known for other things. They will be used to accelerate fracture healing and to stimulate the growth of bone around orthopaedic implants to strengthen their anchorage to the bones and lengthen their life spans. However, this is not all that these versatile peptides can do. They can treat psoriasis and accelerate the repopulation of bone marrow injured by radiation or chemotherapeutic drugs. They may even moderate atherosclerotic and diabetic vascular ossification.

1.1 The parathyroid hormones

There are four potently osteogenic (i.e., anabolic) PTHs either on the market or in clinical trials (Figure 1). One of these is the full-length, 84 amino acid PTH, which has completed its Phase III clinical trial and is called Preos™ by its manufacturer NPS Pharmaceuticals (UT, USA) [1-5]. In order of size, the next one is Lilly's rhPTH-(1-34)OH (also known as teriparatide, a contraction of tetratriacontaparathyroidpeptide), which has been approved for treating osteoporosis by the US FDA and marketed under the name Forteo [1-4]. Then, the two smaller, less well-known potently oestrogenic Ostabolin family peptides, Ostabolin™ (hPTH-[1-31]NH_2) and Ostabolin C™ (i.e., cyclase ostabolin; [Leu27]cyclo Glu22-Lys26 hPTH-[1-31]NH_2) that were first synthesised and characterised at the National Research Council of Canada [6-8]. Ostabolin C has successfully completed its Phase I clinical trial under the auspices of Zelos Therapeutics (Ottawa, Canada). A PTH analogue has been produced by recombinant technology and put into an oral capsule by Unigene Laboratories (NJ, USA) [9]. This peptide is nearly 10-fold better able than hPTH-(1-34)OH (the non-recombinant equivalent of Forteo) to enter the blood of dogs from the Unigene capsule [9]. In an ongoing Phase I trial conducted by GlaxoSmithKline (GSK), an unspecified Unigene analogue can enter the bloodstream of humans in an intact and active form [10].

These four peptides stimulate bone growth in ovariectomised rats, cynomolgus monkeys and osteoporotic postmenopausal women when injected subcutaneously once daily. They can also stimulate bone growth even more effectively when osteoclast generation is blocked by the potent anti-osteoclastogenic osteoprotegerin [11]. The FDA approved rhPTH-(1-34)OH to be self-injected by the patient once daily at a dose of 20 µg for no longer than 2 years. However, cyclic

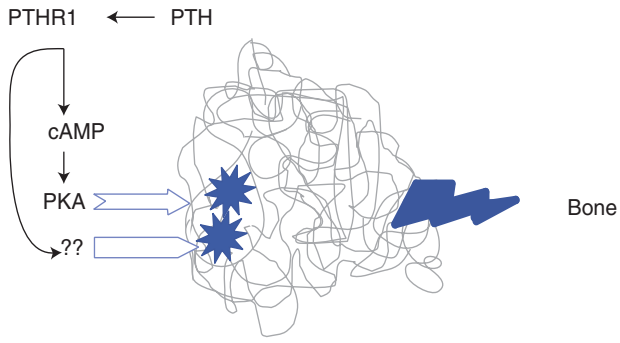

Figure 2. The dense tangle of transmitters, transcription factors, transcripts and translations of the information encoded by the literally hundreds of genes activated by signals from PTH-activated PTHR1 receptors [2]. The stream of signals from the receptors includes a burst of PKA, which is needed to start the bone-building machinery. However, the signal mix and the cascades it triggers depend on what other receptors the PTHR1s are linked to and what signaller-loaded scaffolds, such as NHERF-1 and -2, the PTHR1s are attached to. The upshot of this is a seemingly ever-growing intricate web of responses hidden among which are the actual bone-building drivers. The challenge is to separate the driving 'wheat' from irrelevant 'chaff'.
NHERF: Na^+/H^+ exchange regulatory cofactor; PKA: Protein kinase; PTH: Parathyroid hormone; PTHR: Parathyroid hormone receptor.

or interrupted treatment (3 months on, 3 months off) would be less stressful, cheaper and possibly as effective as continuous treatment [12,13]. Treatment could be much easier for the patient if Unigene/GSK's oral PTH analogue [10] makes it all the way through its clinical trials.

It is important that the PTHs are used only to restore already structurally weakened bone microstructure rather than preventing bone loss after menopause, after immobilising spinal cord injury or during space travel; there are many far cheaper antiresorptive, as opposed to anabolic, drugs for preventing loss, such as the bisphosphonates. Indeed, it would be much wiser to equip initially young, healthy, strong-boned astronaut crews with a state-of-the-art antiresorptive to prevent bone loss.

1.2 How might the parathyroid hormones act on bone?

Unlike the bisphosphonates, such as the widely used alendronate (Fosamax™), which halt or at least slow postmenopausal bone loss and increase bone mineral density by killing osteoclasts and prolonging bone mineralisation by osteoblasts without directly increasing bone matrix mass, these true bone-anabolic PTH peptides directly stimulate bone formation [1-4]. The tangled web of hundreds of genes turned on or off and the dozens of mechanisms stimulated or inhibited by the various signals from PTH-activated PTH receptor 1 (PTHR1) and other associated receptors and signalling complexes in cell membrane rafts has made it extremely hard to spot the definitive osteogenic trigger(s) (Figure 2). However, PTHs work by stimulating the proliferation of early osteoprogenitors without PTH receptors via paracrine or 'missionary' factors, such as fibroblast growth factor-2 (FGF-2) and IGF-I (in rats) or IGF-II (in humans) released by PTH-stimulated, PTHR-loaded osteoblasts; by inducing preosteoblasts with their emerging PTHRs to permanently stop proliferating and differentiate into bone-building osteoblasts and beyond; by making osteoblasts express agents such as Bcl-2 and survivin to prevent them from committing apoptotic suicide and work longer to make more bone; and, last but not least, by inducing retired osteoblasts, the bone lining cells, to temporarily come out of retirement and reversibly re-tool themselves for bone-making [1-4,14] (Figure 3). It follows that such bone-building peptides should also accelerate fracture healing as well as accelerate and enhance the installation of artificial hips and knees.

1.3 Fracture healing

PTHs are prospective accelerators of the mending of even severe non-union fractures (Figure 1). Alkhiary *et al.* [15], Andreassen *et al.* [16-18], Kim and Jahng [19] and Nakajima *et al.* [20] have shown that subcutaneous injections of hPTH-(1-34)OH and [Lys27]cyclo(Glu22-Lys26)hPTH-(1-31)NH_2 significantly accelerate tibial and femoral fracture healing in rats. Bonadio and colleagues [21-24] have gone further and devised a very elegant genomic way of dramatically accelerating fracture healing with hPTH-(1-34)OH. They placed degradable sponges, armed with a plasmid containing DNA encoding hPTH-(1-34)OH, into surgically produced non-union fractures in rat femurs and beagle tibias and femurs that would normally have healed very slowly or not at all. However, the fibroblasts in the fractures' granulation tissues picked up the 'spiked' plasmid and temporarily (hence safely) became microbioreactors, making and secreting hPTH-(1-34)OH just long enough to dramatically drive the complete bridging and healing of these severe fractures.

1.4 Prosthesis anchoring

Besides strengthening and microstructurally restoring ageing osteoporotic bones, and mending broken bones in both the young and old, it seems that the PTHs will also be used to enhance the mechanical interlocking with bone and the load resistance of artificial knees and hips [25-27] (Figure 1). This will benefit the growing number of seniors who need to replace their worn-out hips and knees. The PTHs should also stimulate the vascularisation, repopulation and new bone formation in traumatically or atraumatically produced necrotic bone or in dead bone allografts. The economic and overall benefits of strengthening and speeding up the anchorage of the ~ 500,000 artificial hips implanted each year worldwide and shortening the patients' recovery times should be substantial.

The lifetime of an implanted device depends on how much bone builds around the implant to hold it in place and how tightly it adheres to the implant [25-28]. Ideally, there should be a thick layer of new bone adhering tightly to the

implant surface because when, for example, a hip is stressed by the very large forces normally put on it during walking or running, any spaces between the bone and implant would allow parts of the implant, for example, to rub against its bony envelope and produce 'wear debris', which would collect in the spaces and be phagocytosed by macrophages and other cells. However, these particles cannot be digested and while futilely trying to do so the cells produce cytokines, such as IL-1β, -6 and TNF-α, that drive a cascade of reactions that generate osteoclasts, which degrade the bony anchor. The further loosening produces even more wear debris and eventually forces the device's replacement [25-28].

There are several recent examples of the PTHs' potent implant-anchoring ability in rats. Skripitz *et al.* [29-31] demonstrated the implant-fixing power of hPTH-(1-34)OH by inserting partial models of dead bone allografts – perforated hollow titanium chambers – into the cortices of the proximal tibias of male Sprague-Dawley rats before following the penetration of endosteal cells into the chambers and the growth of bone in the chambers. By 6 weeks the bone in the chambers of the untreated rats had been hollowed out by injury-induced osteoclasts to form a marrow space with only a few trabeculae. However, in the rats that had received daily subcutaneous injections of hPTH-(1-34)OH, the chamber was filled with a thick forest of trabecular struts and plates. It was then shown [29-31] that subcutaneously injected hPTH-(1-34)OH stimulated the fixation of stainless steel screw implants to rat tibias and the attachment of polymethylmethacrylate cement to bone. In only 2 weeks, the PTH injections so strongly stimulated the growth of dense fibrous bone-anchoring tissue around the screws that it took twice as much force to pull them out of the tibias as it took to pull the implants out of the control rats' tibias. Shirota *et al.* [32], using a model relevant to osteopenic and osteoporotic postmenopausal women with prostheses, found that hPTH-(1-34)OH reversed the thinning of bone around titanium screw implants in ovariectomised rats. More recently, Allen *et al.* [33] have reported that ostabolin C is significantly better than hPTH-(1-34)OH at stimulating the growth of bone around a polymethylmethacrylate pin in rat tibias.

However, PTHs have their limits if the implant loosening has gone too far. The grinding of bone against a loose implant, the accompanying surges of microcracking fluid pressure on the enveloping bone, and the osteoclastogenesis-triggering wear particles rubbed off the implant all contribute to the further loosening of the inadequately anchored prosthetic implant. It appears that oscillating fluid pressure is the most potent inducer of peri-implant osteolysis in rats [34]. Astrand *et al.* [34] tested the possibility that the osteoclasts that chew up this bone can be suppressed by etanercept, a competitive inhibitor of TNF-α. However, it didn't prevent the pressure-induced resorption of bone beneath an implanted plate. Nor did the bone formation stimulated by intermittent injections of hPTH-(1-34)OH compensate for the pressure-induced bone resorption, beneath the pressed plate. Only killing osteoclasts with alendronate reduced the bone resorption [35]. Clearly it is important to tightly lock an implanted device in place at the outset with a bone-building PTH to minimise implant sliding, implant-bone grinding and implant-bone collisions before the beginning of bone resorption, which can only be reduced by a bisphosphonate.

All of these reports herald a new era for people needing new joints; obtaining the longest service from an implant requires a PTH peptide to build a lot of new bone around the implant surface without spaces that could fill up with wear debris. Moreover, at the first signs of loosening or even as a periodic prophylactic measure, a subsequent treatment with PTH could give an anchorage-reinforcing boost to the implant.

Obviously the new wall of PTH bone around an implant must be protected from the ravages of osteoclasts generated by pressure from the implant and wear-and-tear debris. This can be achieved, for example, with an oral osteoclast-killing bisphosphonate such as alendronate being taken by the patient for reducing postmenopausal bone loss, or if the implant is coated with calcium apatite, containing a bisphosphonate [35-37].

1.5 Treating psoriasis and baldness

The slowly and/or sporadically cycling stem cells in the basal layers of the epidermis spin off rapidly but limitedly, proliferating transit amplifying progeny, which after a few cycles stop proliferating, lift off the basal layer and are converted into keratin-loaded cadavers by a hybrid process we have called diffpoptosis (differentiation and apoptosis) [38]. The basal stem cells do not have PTH/PTH-related peptide (PTHrP) receptors but cycling transit amplifying basal keratinocytes start making PTH/PTHrP receptors, which, unlike the conventional PTHR1, do not activate adenylyl cyclase although their cellular owners are equipped to do so as indicated by a large burst of adenylyl cyclase activity when they are exposed to the β-adrenergic receptor-activating isoprenaline or to hPTH-(1-34)OH after being made to express conventional PTHR1 [39,40]. The keratinocytes make PTHrP to stimulate these receptors after they stop proliferating and lift off the basal lamina [38]. The amount of PTHrP coming down from the suprabasal cells somehow tells the basal cells how many suprabasal cells are there and accordingly controls the rate of proliferation; a lot of cells above in the stack means a lot of PTHrP coming down and a reduced production of basal cells, whereas a depleted cell stack means less PTHrP coming down and increased basal cell proliferation. However, the PTHrP expression in the lifted cells does not last. When they rise through the granular layer and into the lower corneum, they reach the head of the transepidermal Ca^{2+} gradient (TECG) and hit a wall of external Ca^{2+} that fires their Ca^{2+} sensing receptors (CaRs), the expression of which has risen from a low basal level to a high maximum level in the granular layer [38,41]. The calcium receptor (CaR) signals switch off PTHrP expression and trigger the last stage of diffpoptosis, the conversion of

granular keratinocytes into cornified corpses that, as a last step before dying and desquamating, get rid of their CaRs and pump out the accumulated Ca^{2+} to maintain the head of the TECG and thus prevent the body from losing this Ca^{2+} [38,41]. Thus, the proliferation and differentiation of keratinocytes are controlled by PTHrP and Ca^{2+}.

What causes some basal stem cell daughters to become diffpoptosis-bound transit amplifying cells? The PTH/PTHrP-receptorless stem cells, perched on the tops of dermal papillae, are tethered to each other by homodimers of the transmembrane Delta 1 protein and to the basal lamina by their matricrine (i.e., extracellular matrix-activated and signalling) β_1 integrins [38,42]. When a daughter cell is pushed out of the stem cell niche it turns on a cell cycle counter set at five and its levels of signalling β_1 integrins and Delta 1 start falling and, with them, the strength of the attachments of its descendants to the basal lamina and to other cells [38,42]. Now the binding of an adjacent, still niche-bound stem cell's Delta 1 to the evicted cell's Notch-1/2 receptors starts the differentiation process by activating these Notch-1/2 receptors [42-44]. The evicted cell now starts expressing another Notch activator, Jagged 1, which will progressively increase as the expression of Delta 1 drops in the cell's suprabasal descendants as they rise up to the granular layer [45]. The Notch signalling causes the evicted cell to become a larger, faster cycling transit amplifier (TA) [46] cell with cell cycle genes stimulated by β-catenin saved by integrin-linked kinase (ILK) kinase from destructive turnover activated by the matricrine signalling by the β_1 integrins. However, the Notch signalling has capped the cell's self-replication potential by turning on the cell-cycle counter. Hence, when the cell cycle counter of a TA cell's descendant reaches zero and its basal lamina-tethering, matricrine-signalling β_1 integrins are below a critical density, it changes the parts of its proliferation-silencing Notch–Notch ligand maturation engine, which turns off its cell cycle genes and dismantles the cell cycle machinery. With its mooring lines gone, the cell lifts off the basal lamina, becomes a spinous cell and sets off on its way to becoming a keratin-packed, desquamating cadaver [38]. If Notch signalling should fail, there will be hyperproliferation, deregulated expression of differentiation markers and invasion of once-forbidden suprabasal non-proliferative zones by cycling keratinocytes [43,47].

Although the keratinocyte PTH/PTHrP receptors cannot activate adenylyl cyclase, the dermal fibroblasts' conventional PTHR1 can [48-50]. The stimulation of adenylyl cyclase is the key to PTHrP's role as the prime controller of keratinocyte proliferation and differentiation as indicated by the fact that PTH-(7-34)OH, which cannot stimulate adenylyl cyclase, actually stimulates human and murine keratinocyte proliferation [51]. Therefore, when the PTHrP originating from the suprabasal keratinocytes reaches the dermal fibroblasts, it would stimulate adenylyl cyclase activity and Jagged-1 production as happens in their relatives, hPTH-(1-34)OH-treated bone marrow osteoblasts and cultured rat UMR106 osteoblasts [52,53]. If this Jagged-1 (the skin's most expressed Notch ligand) is the same as the soluble Jagged-1 made by neonatal keratinocytes [54], it would be a paracrine stimulator of the Notch receptors on the overlying keratinocytes that drives their differentiation [42-45,54-57]. Alternatively, the PTHrP-stimulated dermal fibroblasts might make a different paracrine factor that induces overlying basal keratinocytes to make the keratinocyte-specific Jagged-1, which in turn drives keratinocyte differentiation and thus establishes the normal epidermal structure including the TECG.

A failure of suprabasal keratinocytes to express PTHrP could, therefore, have a domino effect on the mechanisms controlling keratinocyte production and differentiation, and this is what happens in psoriatic lesions where PTHrP is not expressed [58]. There is also an underexpressed Jagged-1/Notch mechanism that is normally upregulated in the epidermal basal layer and there is no Ca^{2+} gradient [56,59]. Coupled with the psoriatic cells' defective mutant cGMP-gated Ca^{2+} channels and impaired machinery for responding to external Ca^{2+} and CaR signals by mobilising Ca^{2+} from internal stores, this would restrain diffpoptosis and promote hyperproliferation [60,61].

It follows that a dermal fibroblast-targeting adenylyl cyclase-stimulating PTH, such as hPTH-(1-34)OH, which would bind and activate PTHR1 as effectively as the (1-34) region of PTHrP (e.g., hPTHrP-[1-34]OH), should substitute for the missing PTHrP and restore the normal epidermal structure in a psoriatic lesion. This is in fact what actually happens. Holick *et al.* [62] have reported that putting the adenylyl cyclase-stimulating hPTH-(1-34)OH (i.e., equivalent to Lilly's Forteo) into a percutaneous absorption-enhancing cream (Novasome™) and instructing psoriasis patients to apply 0.1 g of this cream containing 20 µg of the peptide to their lesions twice daily for 2 months restored the normal epidermal structure to formerly recalcitrant lesions. The clue to how the PTH works is the fact that hPTH-(7-34)OH, which could not stimulate adenylyl cyclase in dermal fibroblasts with their conventional PTHR1 receptors, does not affect keratinocyte proliferation [51]. Thus, it is likely that one or more of the adenylyl cyclase-activating PTHs (Figure 1) will be used to treat at least those cases of psoriasis that are resistant to other drugs.

However, as shown above, an adenylyl cyclase-activating PTH such as hPTH-(1-34)OH restrains keratinocyte proliferation and thus can treat psoriasis, but a PTH such as PTH-(7-34)OH that cannot stimulate adenylyl cyclase stimulates the proliferation of human and murine keratinocytes [51]. Holick *et al.* have shown that whereas, as expected, hPTH-(1-34) and PTHrP reduce hair growth, PTH-(7-34)(OH) dramatically stimulates hair growth in C57 Bl/6 mice [51]. This stimulation is probably due to PTH-(7-34)OH binding to PTHR1 and blocking the binding and activation of adenylyl cyclase by endogenous PTHrP. Thus, PTH-(7-34)OH or a more effective analogue may one day be used to treat baldness (Figure 1).

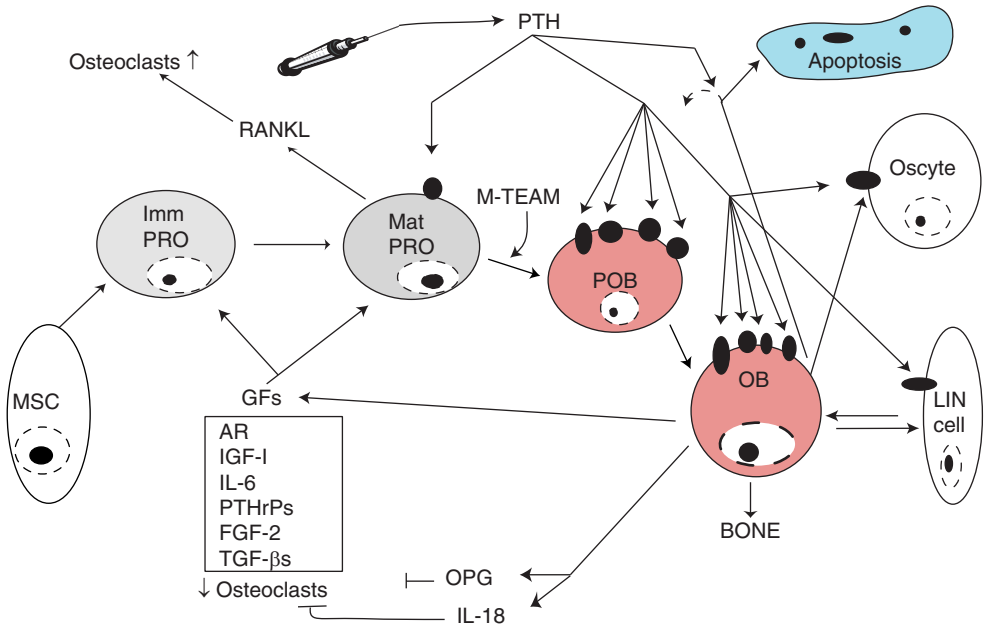

Figure 3. How a subcutaneously injected PTH such as rhPTH-(1-34) may stimulate bone formation [2]**.** The solid ovals on the cells are PTHR1 receptors, the number of which indicates the relative densities that peak as the cells enter the osteoblast stage and then fall if the cells are chosen to be strain-sensing osteocytes or lining cells. The 'M-TEAM' is a group of genes and their products (APRO1, HAX1, Q6, Runx2) that turn off the cell cycle machinery and irreversibly trigger the terminal osteoblast maturation [2]. OPG is a soluble decoy piece of the RANK receptor that prevents RANKL from binding to authentic RANK receptors on osteoblasts and osteoclastic cells. RANKL is the osteoclastogenic factor made by immature osteoblastic cells, which can also stimulate bone growth when attached as oligomers to RANK receptors on osteoblasts [134]. It is important to note that PTHrP is produced by the differentiating osteoblasts and stimulates their and their neighbours' PTHR1 receptors, and is thus one of the fundamental programmed drivers of osteoblastic maturation.
AR: Amphiregulin; FGF: Fibroblast growth factor; GF: Growth factor; IGF: Insulin-like growth factor; Imm PRO: Proliferatively competent immature progenitor cell; LIN: Proliferatively inducible lining; M: Maturation; Mat PRO: Proliferatively mature progenitor; MSC: Proliferatively competent mesenchymal stem cell; OB: Proliferatively shut-down osteoblast; OPG: Osteoprotegerin; Oscyte: Proliferatively shut-down, strain-sensing osteocyte; POB: Proliferatively shut-down preosteoblast; PTH: Parathyroid hormone; PTHR: PTH receptor; PTHrP: PTH-related peptide; RANK: Receptor activator of NF-κB; RANKL: RANK ligand; rhPTH: Recombinant human PTH.

1.6 Stimulating haematopoietic stem cells and restoring damaged bone marrow

In 1961, Rixon and Whitfield [63] reported that injecting 50 – 200 USP (a unit to measure the mass of a vitamin/drug or its expected biological effects) units of Lilly's bovine parathyroid extract 5 min – 1 h after whole-body irradiation with 8 Gy of 2000 kilovolt peak (kvp) x-rays increased the average 30-day survival of 1296 300-g male hooded rats from 33 – 73% ($p <<< 0.001$). This striking effect at this dose level indicated that PTH somehow reduced radiation-induced apoptotic cell killing and/or increased the proliferation of surviving haematopoietic stem cells in the rat bone marrow. From what is now known, it seems that the PTH injections worked at least partly by preventing apoptosis of irradiated haematopoietic stem cells just as they do in osteoblastic cells [64] (Figure 3).

In 1971, Perris *et al.* [65] presented PTH as a key controller of haematopoiesis in the rat. And then Gallien-Lartigue and Carrez [66] confirmed this when they showed that PTH indeed stimulated the proliferation of haematopoietic colony-forming unit spleen (CFU-S) stem cells in mice. Calvi *et al.* [52] and Weber *et al.* [53], ~ 30 years later, reported that hPTH-(1-34)OH, the potent adenylyl cyclase-stimulating forskolin or the continuous stream of signals from a permanently switched-on mutant PTHR1 receptor, which does not need PTH or PTHrP to activate it, can enlarge the haematopoietic stem cell pool in mice. The cAMP signals from activated PTHR1 cause the osteoblastic bone-lining cells to make large amounts of surface Jagged 1, which binds to Notch receptors (see Section 1.5) in this case on adjacent haematopoietic stem cells attached by N-cadherin to the osteoblastic lining cells [52,53,67-69]. Unlike the terminal differentiation-driving Notch signals in keratinocyte stem cells, the resulting Notch signals in the haematopoietic stem cells restrain differentiation, thus increasing the size of the stem cell pool. Weber *et al.* [53] have subsequently shown that intermittent PTH injections into mice, after marrow ablation and bone marrow transplantation with limited, marginally sufficient numbers of donor cells, dramatically improved the survival of the animals and increased the bone marrow cellularity in their hind limbs.

It now seems likely that adenylyl cyclase-activating PTH peptides can be used to promote the restoration of bone marrow depleted by radiation or chemotherapeutic agents by

stimulating the expression of antiapoptosis proteins, such as Bcl-2 and survivin, in endogenous marrow stem cells and stimulating the expansion of marrow stem cell populations. The PTHs would also accelerate the repopulation and functional restoration of damaged marrow by transplanted bone marrow cells.

1.7 Preventing macrovascular ossification

Ectopic bone can form in the blood vessels and heart valves of atherosclerotics or diabetics with the aorta and the cardiac valves being the most commonly affected [70-74]. There are three kinds of macrovascular calcification or ossification; atheroclerosis, calcific valvular sclerosis and medial artery calcification or ossification [74]. These are not as harmless or as rare as they were once thought to be. Indeed, the stiffness of ossified aorta and coronary arteries is a major contributor to myocardial infarction and heart failure, and medial arterial ossification is responsible for the vascular damage leading to limb amputation in Type 1 and 2 diabetics [70,71,74,75]. Although unappreciated until recently, vascular ossification has been known for a long time. As long as 300 years ago diseased coronary arteries were reported to have changed into bony canals [72]. In 1863, Virchow said that the plates that pervade the inner wall of such blood vessels are not just calcium phosphate precipitates but real plates of bone; "we see ossification declare itself in precisely the same manner as when an osteophyte forms on the surface of bone…the osteophytes of the inner table of the skull…follow the same course of development as the ossifying plates of the internal coat of the aorta and even of the veins" [76]. A drug that can stop ossification would obviously help to prevent heart failure and diabetic limb amputations. On the other hand, a drug that promotes vascular ossification is dangerous and should be discarded. As the PTHs are potent stimulators of skeletal bone formation it is important to question whether they also stimulate vascular bone formation.

There are two kinds of cardiovascular ossification mechanism just as there are two main forms of bone formation; membranous and endochondral. First is cardiac valve ossification and the medial vascular ossification of diabetes, which, as Virchow pointed out in 1863 [76], resemble the cartilage-independent membranous bone formation [73]. The other is atherosclerotic ossification, which resembles cartilage-dependent endochondral bone formation [73] and will be the focus of what follows.

Atherosclerotic ossification starts with the accumulation and retention of low-density lipoprotein (LDL) complexes in the lesion-prone region of a coronary artery's wall where their lipids are oxidised and apolipoproteins glycated [77-79]. The modified LDLs signal the endothelial cells to put molecules such as E-selectin on their surfaces to snare passing monocytes and T lymphocytes and lure them into the vascular tunica intima. There, the monocytes become macrophages, which pick up the LDLs using their 'scavenger' receptors and become fat globule-loaded 'foam cells', produce pro-inflammatory cytokines such as TNF-α that stimulate the T cells and vascular smooth muscle cells (VSMCs) in the tunica media to make osteoinductive factors such as bone morphogenetic protein-2 (BMP-2) [77-80]. The macrophages and endothelial cells also make BMP-2. The VSMCs migrate from the media to beneath the endothelial lining to thicken the intima and build a relatively fragile matrix canopy over the nest of foam cells and T cells. The swelling mass of foam cells in this roiling nest eventually make enough matrix metalloproteinases to cut through the cover and cause an artery-blocking clot or embolism [77-80].

The BMP-driven, osteogenically competent cells in the tunica media of a blood vessel are adventitial myofibroblasts (pericytes), which are directed on to the osteoblast maturation pathway mainly by BMP-2–Msx2-driven mechanism as happens in the flat cranial bones [74]. However, the osteogenically redifferentiation-competent subpopulation of the coronary artery's proliferating VSMCs (calcifying vascular smooth muscle cells [CVCs]) are steered onto the road to cartilage and from there to vascular invasion of the cartilage and finally bone mainly by the BMP-2–Cbfa1/Runx2- and Sox 9-driven, cartilage-mediated mechanism as happens in the PTHrP-controlled formation of a long bone such as the femur [73-76,81-92]. This large lesion can become a complete bony microcosm even with its own blood vessels and bone marrow.

As VSMCs and vascular endothelial cells make the osteogenic PTHrP and the PTHR1 receptors the PTHrP's N-terminal region shares with the osteogenic PTHs [2,93], hPTH-(1-34)OH or an N-terminal PTHrP fragment such as hPTHrP-(1-34)OH obviously should stimulate vascular ossification. However, Jono *et al.* [88] were the first to report that PTH-(1-34)(OH) and the PTHrP-(1-34)(OH) fragment surprisingly decreased calcification by bovine vascular smooth muscle cells and used both their adenylyl cyclase and protein kinase C to do so. Ishikawa *et al.* [93] later showed that PTHrP is made by VSMCs and maybe by the macrophage foam cells in human and rat atherosclerotic lesions. By reducing VSMC proliferation and their migration to the foam cell nest in the intima, the autocrine/paracrine PTHrP serves as a protective, antiatherogenic and, thus, antiossification cytokine. Shao *et al.* [94] reported that intermittent injections of hPTH-(1-34)OH into LDL receptor $(LDLR)^{-/-}$ mice prevented a diabetogenic high-fat diet from ossifying aortae and heart valves as it did in untreated control animals. The peptide apparently did this at least in part by increasing the production of soluble and probably phosphorylated osteopontin in skeletal cells, but not by the vascular cells [94]. The circulating osteopontin from the PTH-stimulated skeleton prevented the vascular osteoprogenitors from expressing their osteogenic Msx2 genes. According to Wada *et al.* [92] the osteopontin from the PTH-stimulated skeleton may also have interfered with apatite crystal formation by interacting directly with the apatite crystal surfaces.

Of course, this is not the whole story; the vascular actions of full-length (holo) PTHrP and its PTH-like N-terminal fragments are very different [95-97]. The VSMCs in the shoulder region; the inflammation action centre; of human carotid atherosclerotic plaques have been found to hyperexpress the autocrine/paracrine/intracrine PTHrP and the PTHR1 receptor, which it shares with the PTHs [95]. The cAMP and its dependent protein kinase A (PKA) signals from the activated PTHR1 receptors stimulate the VSMCs to make monocyte chemotactic protein-1 (MCP-1), a chemokine peptide that stimulates circulating monocytes to migrate through the vascular endothelium and join the inflammation-driving macrophages in the vascular intima [95]. Thus, the VSMCs holoPTHrP drives the loading of the intima with pro-inflammatory and osteoinductive factors.

However, the holoPTHrP made and secreted by the VSMCs in the plaque shoulder has something that the PTHs in Figure 1 do not have; a nuclear/nucleolar localising signal (NLS) that enables the translational holoPTHrP product to be shipped from its synthesis site in the VSMC's endoplasmic reticulum into the nucleus [2,98-100]. Thus, holoPTHrP is an intracrine as well as autocrine and paracrine cytokine. The holoPTHrP translation product with a traditional upstream signal sequence is grabbed by the cells secretion machinery as it emerges from the endoplasmic reticulum, is secreted and with its N-terminal 1-36 region activates the producer cells or its neighbouring cells PTHR1 [2,98-100]. But there are other translation initiation sites, the use of any one of which disrupts the signal sequence and prevents the translational product from being grabbed by the secretory mechanism [2,98-100]. This emerging holoPTHrP is instead dumped into the cytosol where its NLS binds to β-importin, which engages the trans-nuclear pore transport mechanism that ships the protein into the nucleus where it stimulates proliferation by a process requiring its C-terminal 112 – 139 region that somehow hyperphosphorylates and, thus, switches off the inhibitory G1/S cell cycle checkpoint regulator retinoblastoma protein (pRb) [99]. Engineering VSMCs to overexpress a NLS-deleted PTHrP fragment, which cannot get into the nucleus to trigger pRB hyperphosphorylation, is instead secreted and, when outside the cell, stimulates PTHR1, the signals from which inhibit VSMC proliferation and vascular ossification as shown by Jono *et al.* [88] and Shao *et al.* [94], and also inhibit the carotid intimal hyperplasia and thickening triggered by angioplasty [98-100].

Thus, the osteogenic PTHs (Figure 1) do not enter the VSMC nucleus to stimulate proliferation and the thickening of the vascular intima. However, the cAMP/PKA signals from the PTHR1 receptors they activate would still stimulate MCP-1 expression and the migration of monocytes into the intima. As yet unknown is what a stimulation of such things as MCP-1 by a PTH like hPTH-(1-34)OH would do to already formed vascular bone. And there could be a problem with the large, full-length rhPTH-(1-84)OH; its C-terminal region (24 – 84) activates the C-terminal PTHR (CTPTHR), which has been found on various cells [101]. This raises the important questions of whether this receptor is also on VSMCs and, if so, do signals from it, when activated by injected rhPTH-(1-84)OH, promote ossification? However, existing bony vascular lesions may be reduced or eliminated by bisphosphonates, which recovering osteoporotics may use to protect their new 'PTH bone' after their PTH treatment [1-4,102]. It is also an unexpected although well-known fact that these potent antiresorptives that prevent skeletal bone cause vascular bone loss [102,103]. It appears that the bisphosphonates bind avidly to blood vessel walls and may kill the atheromatous lesion's macrophages that would become the foam cells that produce the pro-inflammatory cytokines such as TNF-α that drive vascular osteogenesis [77,78,102].

1.8 Parathyroid hormone cure-alls and their widespread receptors

By now, the reader might suspect that the PTHs can treat all afflictions in man or beast. However, the variety of the PTHs' clinical targets is due to many different cells making PTHrP and having PTH/PTHrP receptors. There are two reasons for this; the first is the ancient invention of PTHrP to drive the mesenchymal–epithelial interactions needed for the development, maintenance and function of over a dozen tissues such as bone, colon and skin [2]. The second reason is the sharing of PTHR1 by the N-terminal region of PTH and the short PTH-like N-terminal region of PTHrP [2].

2. Leptin

Leptin has been called 'the voice of the adipose tissue' [104]. Its circulating level is a measure of the body's energy stores. It is a 16.7-kDa peptide member of the cytokine superfamily which, from studies on leptin-lacking obese mice with disabled leptin genes, was originally believed to be made only by white fat cells and restrain over-eating by stimulating target neurons in the hypothalamic arcuate nucleus [2,105,106]. It is now known to be made by many other cells including bone, cartilage and vascular cells. Thus, leptin is emerging as a versatile player in many different bodily functions. One of its recently discovered roles is helping to maintain the body's overall adrenergic tone and, therefore, blood pressure by stimulating neurons in the ventromedial hypothalamic (VMH) nuclei that project to presympathetic neurons in the lateral columns of the spinal cord and from them to the adrenergic nerves throughout the body including the bones and blood vessels (Figure 4) [2,107,108]. Therefore, the large amounts of circulating leptin in obese people are likely to be responsible for their obesity-associated hypertension [109].

Leptin's hypothalamus-mediated actions affect bone in two ways. First, the cytokine keeps the ovaries cycling and producing the oestrogen pulses without which bones deteriorate and cause osteopoenia in ovariectomised rodents and osteoporosis in postmenopausal women [1-4,105]. Second, leptin restrains

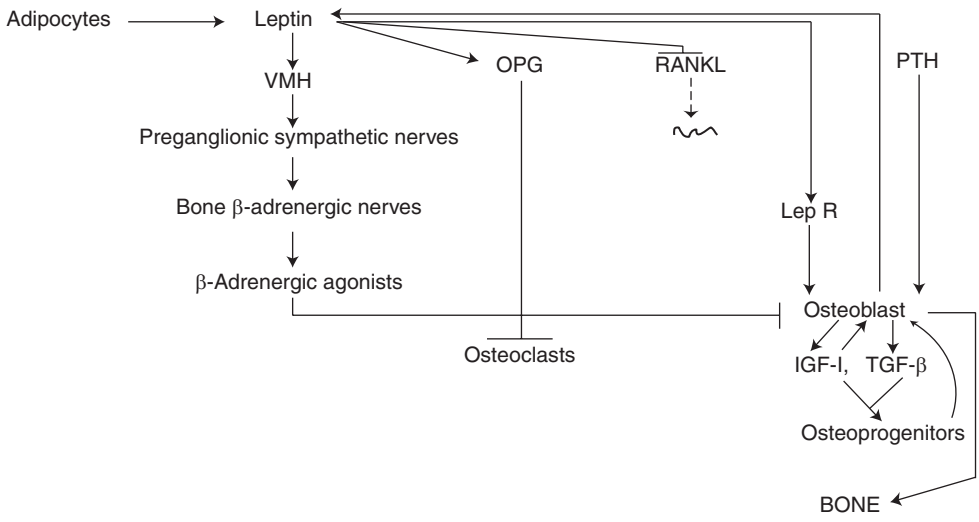

Figure 4. The indirect negative control of bone growth by leptin via its adrenergic system driving neuronal targets in the VMH nuclei and the direct positive, hypothalamus-independent control of bone growth by the cytokine and its receptor. Leptin from fat cells, osteoblasts and even brain cells, or injected intracerebroventricularly, stimulates receptor-bearing neurons of the VMH nuclei. These neurons project to the preganglionic sympathetic neurons of the lateral cell columns of the spinal cord and these in turn project to the bones' β-adrenergic nerves, which fire their β-adrenergic transmitters at osteoblasts with β_2-adrenergic receptors on their surfaces.

IGF: Insulin growth factor; LepR: Leptin receptor; OPG: Osteoprotegerin; PTH: Parathyroid hormone; TGF: Transforming growth factor; VMH: Ventromedial hypothalamic.

bone growth by targeting neurons in the VMH (Figure 4) [107,108]. This latter inhibitory action was demonstrated by injecting leptin directly into the third cerebral ventricle of a mouse and it sparked the incorrect idea that the cytokine is an osteoblast suppressor [5].

Leptin's VMH/adrenergically mediated antiosteogenic activity suggested that β-adrenergic agonists such as clenbuterol, isoprenaline and salbutamol should reduce osteogenic activity, whereas β-adrenergic antagonists such as propranolol should stimulate it. This is indeed what has been found in mice [107,108,110-112]. Moreover, propranolol enhances the stimulation of vertebral trabecular growth in mice by hPTH-(1-34)OH [112]. Furthermore, taking β-adrenergic blockers reduces bone fracturing in women, which, together with the data from mice, has suggested that these drugs may be used to treat osteoporosis [107,108,113].

Although leptin indirectly suppresses osteoblasts, it really should be an anabolic agent. This would be expected because a large drop of the circulating leptin level means starvation and the need to shut down rather than stimulate such energetically expensive, temporarily dispensible processes such as reproduction and bone growth, whereas replenishing the body fat stores should restore the cytokine's level and restart these costly ATP-consuming processes [2,114,115].

The response to a lack of leptin in mice depends on the part of the skeleton that is being focussed on. The leptin lack in Ob(Lep)$^{-/-}$ mice increases the bone-mineral content, bone mineral density (BMD), trabecular bone volume and the length and breadth of the lumbar vertebrae, whereas it shortens the femurs and reduces their bone mineral content, BMD, cortical thickness, trabecular thickness and trabecular volume [116]. Hamrick *et al.* [116] have suggested that this difference may be due to the chondrocytes of the vertebral growth plates responding differently from femoral growth plates; it may be that they are more sensitive to, or more controlled by, leptin and VMH-dependent β-adrenergic activity. The loss of long-bone mass is likely to be due partly to severely reduced loading because of a significant drop in muscle (e.g., quadriceps) mass as well as the basic inactivity of these obese animals. Another contributor to the loss of femoral bone mass is a reduction of the expression of TGF-β, a stimulator of osteoprogenitor cell proliferation and an anti-adipogenesis agent, which causes a massive increase in adipocytes and a drop in the femoral osteocyte population [116].

Peripherally, as opposed to intracerebroventricularly, injected leptin or the leptin from fat or bone cells themselves directly stimulates bone growth first by pushing marrow stromal cells off the adipocyte pathway and on to the osteoblast road, then by stimulating the mineralising osteoblast–osteocyte transition, and also by inhibiting osteoclastogenesis by suppressing the osteoclastogenesis-stimulating receptor activator of NF-κB ligand (RANKL) peptide and stimulating the expression of the osteoclastogenesis suppressor, osteoprotegerin (Figure 4) [118-124]. Leptin's osteogenic activity resembles that of the PTHs [110]. The resemblance may be even closer because PTHrP stimulates lung lipofibroblasts to make leptin [125]. Therefore, leptin may at least partly mediate the PTHs osteogenic actions (Figure 4).

Of course, the osteogenic ability of leptin raises the question of whether there is a relationship between plasma leptin concentration and the fragility and loss of bone in postmenopausal osteoporotic women. Yamauchi *et al.* [126] found that plasma leptin and BMD levels were significantly and positively correlated, and the leptin and fracture incidences were negatively correlated in 139 postmenopausal women with an average age of 62.5 years. Roux *et al.* [127] found that the plasma leptin level tends to correlate positively with BMD and negatively with a bone resorption marker (C-terminal crosslinking telopeptide of type I collagen) in the hips and spines of healthy postmenopausal women. However, others [128-131] have found no relationship between BMD and serum bone markers in postmenopausal women.

The similarity of leptin's osteogenicity to that of the PTHs raises the question of whether the vascular wall is also a leptin target and how it may affect vascular bone formation. It appears that the subpopulations of osteogenically competent VSMCs and pericyte osteoprogenitor cells do have leptin receptors and that, unlike hPTH-(1-34)OH, the cytokine can stimulate their differentiation into calcifying, bone-building osteoblasts and, because of this, causes a decrease in vascular distensibility just as it drives the differentiation of bone marrow osteoprogenitors [132,133]. This plus leptin's stimulation of hypertensive adrenergic activity makes it a potentially very dangerous bone-anabolic drug.

3. Expert opinion and conclusions

One of the adenylyl cyclase-stimulating PTHs is the first true potent bone-building, as opposed to antiresorptive, drug to be used to treat osteoporosis, but there will soon be other PTHs. However, osteoporosis is just one of the many things these versatile peptides will sooner or later be treating via their body-wide PTHR1 receptors. They will also be used for other skeletal tasks, such as speeding up the mending of severe non-union fractures and strengthening and reinforcing the anchorage of orthopaedic devices to bone. They will also be used to treat psoriasis and perhaps to speed up the regeneration of bone marrow depleted by radiation and chemotherapeutic agents and retard vascular calcification (especially when used along with bisphosphonates).

Leptin is another member of the now growing family of bone-anabolic peptides. However, it is unlikely to have much of a future because the value of its PTH-like osteogenic potency is compromised by its promotion of hypertension by stimulating hypothalamus-driven β-adrenergic activity and its un-PTH-like promotion of vascular calcification. However, a spin-off from the leptin studies is the discovery that adrenergic antagonists such as propanolol are osteogenic and have suggested to some that they may be used to treat osteoporosis. However, it seems unlikely that β-adrenergic antagonists with their potential for cardiovascular side effects could compete with the potently osteogenic, as well as very versatile and safe, PTHs.

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