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Extracellular Nucleotide Signaling: A Mechanism for Integrating Local and Systemic Responses in the Activation of Bone Remodeling

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Bone turnover occurs at discreet sites in the remodeling skeleton. The focal nature of this process indicates that local cues may facilitate the activation of bone cells by systemic factors. Nucleotides such as adenosine triphosphate (ATP) are locally released, short-lived, yet potent extracellular signaling molecules. These ligands act at a large family of receptors—the P2 receptors, which are subdivided into P2Y and P2X subtypes based on mechanism of signal transduction. Nucleotides enter the extracellular milieu via non-lytic and lytic mechanisms where they activate multiple P2 receptor types expressed by both osteoblasts and osteoclasts. In this review the release of ATP by bone cells is discussed in the context of activation of bone remodeling. We provide compelling evidence that nucleotides, acting via P2Y receptors, are potent potentiators of parathyroid hormone-induced signaling and transcriptional activation in osteoblasts. The provision of a mechanism to induce activation of osteoblasts above a threshold attained by systemic factors alone may facilitate focal remodeling and address the paradox of why systemic regulators like PTH exert effects at discreet sites. (Bone 28:507–512; 2001)

Key Words: Adenosine triphosphate (ATP); Osteoblast; P2 receptor; Parathyroid hormone (PTH); Remodeling; c-*fos*.

Introduction

The quantum concept of remodeling in which discrete microscopic packets of bone are removed and replaced throughout the skeleton is now recognized as the predominant form of turnover in the adult skeleton. The primary event in remodeling is activation, during which lining cells retract from the bone surface to allow access of osteoclasts or their precursors to the underlying mineralized matrix. The regulation of activation is poorly understood but the focal nature of remodeling indicates that this process is sensitive to local stimuli, including mechanical strain. In addition, systemic factors, in particular parathyroid hormone (PTH), are known to increase the rate of activation (for review see Mundy[54]). Thus, remodeling at foci involves the combined action of systemic factors and local cues. In this review, we suggest that one important local stimulus may be obtained from extracellular nucleotides. They can exist locally within the bone microenvironment to initiate intracellular signaling via P2Y receptors, which sensitizes cells to the action of PTH and thus is one mechanism for integrating local and systemic responses in bone. The key observations are:

- Cells of the osteoblast and osteoclast lineages express functional P2 receptors.
- Activation of P2 receptors results in the induction of c-*fos*, a transcription factor that plays a pivotal role in the regulation of bone remodeling.
- Extracellular nucleotides exhibit a potent synergy with PTH, enhancing intracellular calcium release ($[Ca^{2+}]_i$), downstream signaling, and gene activation.
- Bone cells release adenosine triphosphate (ATP) into the extracellular environment and the release is enhanced by mechanical strain and regulatory factors.

The concept that nucleotides can act in the extracellular environment as potent signaling molecules is not new. The ability of ATP to effect neurotransmitter responses from certain nerve terminals was first proposed by Burnstock over 20 years ago.[16] Since that time, a large family of receptors has been defined, both pharmacologically and molecularly, which transduce signals arising from nucleotide stimulation.[6,26] These P2 receptors (formerly termed purinoceptors) have been subdivided into two classes: P2Y, comprised of five distinct receptors coupled to heteromeric G proteins; and P2X, containing seven distinct receptors that function as cationic gated channels. Physiologically, P2X receptors are activated exclusively by adenine nucleotides, whereas P2Y receptors are responsive to adenine or uridine nucleotides or, in some cases, to both. P2 receptors are expressed ubiquitously, but specific tissue responses are achieved by cell-specific expression profiles. Receptors are coupled to multiple specific cellular functions in processes as diverse as neurotransmission, wound healing, morphogenesis, and apoptosis (reviewed in Burnstock[17]).

There is now conclusive evidence that nucleotides are important local signaling molecules in bone.[11,22,41,77] Both

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osteoblasts and osteoclasts are targets for nucleotide activation through multiple P2 receptor types.[4,10,28,47,55,60,75] These receptors couple to multiple signal transduction cascades, including inositol triphosphate (IP_3)-mediated intracellular calcium release (P2Y receptors) or nonselective outward cation currents (P2X receptors). These signaling cascades, dependently and independently of mitogen-activated protein kinase activation, induce expression of genes such as c-*fos*,[11] a transcription factor—the importance of which, in the early regulation of remodeling processes, is reflected in the skeletal pathologies associated with its deletion[73] or overexpression.[40] Downstream of these signaling events nucleotides have been reported to modulate osteoblast-induced bone formation[41] and proliferation,[63] as well as modulate osteoclastogenesis and bone resorption.[4,14,53] These processes have recently been succinctly reviewed by Dixon and Sims;[22] therefore, in this review we focus on describing how locally released nucleotides, through P2Y receptor activation, may modulate the signaling of systemic PTH.

Sources of Extracellular Nucleotides in Bone Microenvironment

For P2 receptors to modulate the activity of bone cells, ATP must exist at a sufficient concentration in the surrounding microenvironment to initiate receptor activation. All cells contain high intracellular ATP concentrations (1–5 mmol/L), as well as the capacity to release ATP following trauma. In addition, increased blood flow associated with the general inflammatory response could greatly increase the nucleotide concentration available upon platelet aggregation. Hence, at sites of tissue injury, wounding, or fracture, high local ATP concentrations will be present to activate P2 receptors. Nevertheless, it is obvious that nucleotides must exist transiently in the bone microenvironment without cell damage to be physiologically relevant regulators of bone remodeling. To investigate whether cells of bone can provide extracellular nucleotides through nonlytic release, we designed a real-time detection system that relies on the high-yield chemiluminescent reaction generated by luciferin and luciferase in the presence of ATP.[19,45] This allowed us to demonstrate that human osteoblasts constitutively release ATP into their extracellular environment (in this case, the surrounding chamber medium or bulk phase) in the nanomolar range.[12,13] Released ATP probably reaches much higher concentrations in the vicinity of the membrane, because the presence of nucleotidases at the cell surface, membrane trapping, and unstirred layer effects will all influence bulk phase measurement. This concept was confirmed in a recent study in which membrane-anchored luciferase revealed micromolar ATP concentrations at the surface of platelets.[5]

In addition to receptors responsive to ATP, osteoblasts express $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors[9,51] (see later), which are preferentially or selectively activated by uridine nucleotides. Recent studies have demonstrated conclusively that cells constitutively release uridine triphosphate (UTP) into their extracellular environment, providing locally released agonist capable of activating these P2-receptor family members.[48]

It is not clear why cells are so "leaky" with respect to nucleotides. One hypothesis proposes that this may be a means of regulating ATP concentration at the plasma membrane, where it is required for the activation of ATP-dependent channels and enzymes.[61] More recent studies suggest that nucleotide release, through autocrine signaling, represents a universal key determinant in establishing the "setpoint" for activation of signal transduction pathways.[58] It would appear that nucleotide release is fundamental in defining basal cellular activity. In fact, the word "leaky" is misleading, as simple diffusion along a gradient cannot account for this observed effect. Sulfonylureas such as glibenclamide effectively inhibit the process both in osteoblasts[13] and other cell types.[34,61,62] This has led to the suggestion that ATP binding cassette proteins, a large family of membrane transporters that includes the sulfonylurea receptor, cystic fibrosis transmembrane conductance regulator (CFTR), and MDR, are the active mediators of ATP release.[32] This remains controversial, with some investigators suggesting that ABC transporters may represent only one component of a larger macromolecular complex.[31,32,62]

Further evidence for a distinct, nonlytic mechanism of nucleotide release lies in the observation that it can be regulated positively (and presumably negatively).[7,13] In a positive feedback loop, UTP acts through $P2Y_2$ receptors to upregulate ATP release from primary human osteoblasts.[13] In addition, ATP and UTP release is modulated positively by fluid flow.[12,48] Indeed, recent evidence suggests that nucleotide release occurs as a consequence of fluid forces generated following cellular manipulation in almost all in vitro experimental systems.[48,58] The importance of this finding becomes particularly apparent in the context of the bone microenvironment, where shear forces resulting from fluid flow within trabeculae and canicular spaces are thought to transmit mechanical forces.[38,46,59,70] Taken together, these observations indicate that a cellular mechanism exists to release ATP into the extracellular environment at concentrations sufficient to activate cell surface P2 receptors, and that this release can be modulated by mechanical and agonist stimulation (**Figure 1**).

Osteoblasts Are Targets for Extracellular Nucleotides

The ability of ATP and adenosine diphosphate (ADP) to initiate phosphatidylinositol-4,5-bisphosphate and inositol-(1,4,5)-trisphosphate [$Ins(1,4,5)P_3$] hydrolysis by phospholipase C (PLC), and thereby release of Ca^{2+} from intracellular stores in the rat osteosarcoma cell line UMR-106, provided the first evidence for P2Y receptor expression by osteoblasts.[47,60] Subsequently, Shoefl et al. demonstrated nucleotide-induced $[Ca^{2+}]_i$ release in primary osteoblasts derived from explants of human trabecular bone.[64] The first molecular evidence for the expression of P2 receptors by osteoblasts came with the cloning of the human $P2Y_2$ receptor from an osteoclastoma cDNA library and its localization to both osteoblasts and osteoclasts.[10] More recent studies from our laboratory and others have demonstrated that osteoblasts express mRNAs for all characterized P2Y receptor types.[14,36,51] This receptor profile varies between primary cells and established osteoblastic cell lines, as well as between different cell lines.[9,11,47,51,60] To add further complexity, there is clear heterogeneity within single populations of both primary and clonal osteoblasts.[21] These differences are almost certainly differentiation-dependent, as reported previously for the progression of human myeloid progenitors.[52] Although these observations are intriguing, their exact significance remains unclear. It is tempting to speculate that P2Y receptor types couple to distinct intracellular signaling systems and thereby effect discrete osteoblast processes. This has proven difficult to address experimentally. Despite differences in the receptor profile between cells, they still express multiple receptor types that are capable of binding and differentially responding to nucleotides. Dissection of individual receptor responses has further been hampered by the lack of specific P2Y receptor antagonists, along with the presence of enzymes that rapidly interconvert or degrade nucleotides.[33]

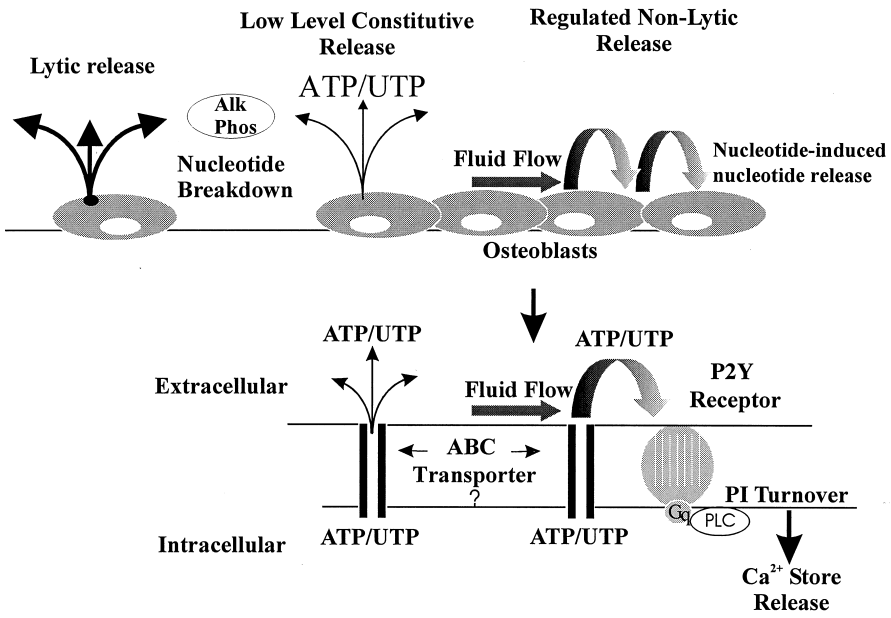

Figure 1. Nucleotides exist in the microenvironment of bone to initiate signaling via P2Y receptors. Nucleotides are released at sites of cell trauma via lytic mechanisms. Low-level constitutive, nonlytic release also occurs, which is proposed to maintain a basal level of cellular activation. This release can be positively modulated by mechanical/fluid forces and in a positive feedback loop, via nucleotide-induced nucleotide release. Nucleotides once released from a cell are rapidly hydrolyzed by nucleotidases and phosphatases, such as alkaline phosphatase, into a range of constituent molecules that includes nucleotide diphosphates, which further signal through a complement of P2 receptors. The mechanism of nonlytic nucleotide release is thought to involve members of the ATP binding cassette protein family of transporters, or associated channels. P2Y receptors expressed by osteoblasts couple through Gq to phosphoinositide turnover, and hence autocrine/paracrine activation by nucleotides leads to calcium release from intracellular stores.

ATP in the Bone Microenvironment

Once released, ATP will be rapidly broken down by ectonucleotidases that abound on the extracellular side of osteoblast membranes.[18] Consistent with the expression of both ecto-NTP pyrophosphatase and alkaline phosphatase, the half-life for ATP released from human SaOS-2 osteosarcoma cells is approximately 50 sec (Bowler et al., unpublished observation). This short half-life restricts the action of ATP to that of a localized signal. However, the ability of nucleotides to induce their own release provides a mechanism for sustaining and possibly propagating a more widespread response.[8,13] ATP release and subsequent paracrine signaling in osteoblasts might provide a mechanism for cell-to-cell communication independently of gap junctions.[42] As suggested earlier, activation of P2Y receptors leads to IP_3-mediated $[Ca^{2+}]_i$ release. In a study by Osipchuk et al., nucleotide-activated cellular activity was visualized as a wave of mobilized $[Ca^{2+}]_i$ spreading outward through the cell population from a single point of ATP/receptor contact.[57] This mechanism would seem to be important in cells and tissues that lack communication via gap junctions, such as chondrocytes and cartilage. In agreement with this model, chondrocytes abundantly express P2Y receptors[49] and have the capacity to release ATP.[50]

Elevated calcium levels can activate a variety of intracellular signaling systems in different cell types. The question is which ones are activated in osteoblasts upon P2Y receptor stimulation? One is clearly the extracellular signal-regulated (ERK) cascade, because this mitogen-activated protein kinase (MAPK) pathway is induced equipotently by ATP and UTP through the $P2Y_2$ receptor in many cell types.[2,66,67] The mechanism by which this occurs in osteoblasts remains to be elucidated. In neuronal cells, several different pathways are implicated. One arises from Ca^{2+}-dependent activation of the tyrosine kinase, PYK2, which then phosphorylates the EGF receptor, leading to recruitment of the Grb2/SOS complex, and thereafter activation of the RAS/RAF/MEK/ERK signaling cascade.[66,67] Alternatively, Ca^{2+} can also target RAS through a distinct pathway involving the exchange factor RAS-GRF.[25] In contrast, in endothelial cells, an integrin-dependent, PYK2-independent and growth factor (GF)-receptor-independent pathway is induced downstream of mobilized $[Ca^{2+}]_i$.[65] Most proliferative signals drive a rapid and robust activation of the ERK pathway, transcription factor phosphorylation, and immediate early gene transcription, particularly the protooncogene c-*fos*.[35] However, nucleotides alone weakly stimulate c-*fos* gene expression and osteoblast proliferation.[11,63] It therefore seems likely that $P2Y_2$-coupled ERK activation couples to additional downstream effectors in osteoblasts. In agreement with this idea, the $P2Y_2$-ERK pathway leads to phospholipase A_2 activation, arachidonic acid production, and prostaglandin E_2 (PGE_2) release from MDK cells,[76] whereas, in MC3T3-E1 cells, ATP activates phospholipase D to stimulate PGE_2 release.[74] These findings are particularly relevant in bone, where prostaglandins have profound effects on osteoblast proliferation[44] and bone formation.[72]

In contrast to $P2Y_2$ receptors, activation of osteoblast $P2Y_1$ receptors via ATP and ADP does not induce the ERK pathway,[11] but does induce phosphorylation of the transcription factor CREB on Ser-133.[29] Although a number of kinases can mediate CREB phosphorylation, calcium-dependent calmodulin kinases (CaMKs) are the most likely candidates downstream of the $P2Y_1$ receptor. This engenders only a weak induction of c-*fos* mediated through the Ca/CRE located just upstream of the TATA box in the c-*fos* promoter.[11] The signaling properties of the $P2Y_4$ or $P2Y_6$ receptors expressed by osteoblasts are unclear; in other cell types these receptors couple to Gq and signal via IP_3 mobilization.[56] The role of these receptors in vivo is unclear, but their presence on osteoblasts would provide a pyrimidine-specific receptor capable of responding to UTP and UDP released into the bone microenvironment.

Nucleotides as Potentiators of Systemic Responses

It has become increasingly apparent to us that extracellular nucleotides serve important roles as costimulators in bone. More specifically, locally released extracellular nucleotides might cooperate with systemic factors to activate signaling in osteoblasts beyond a key threshold that neither stimulus alone could attain. This evidence has stemmed from many early reports showing that extracellular nucleotides potentiate GF-induced proliferative responses in cells of stromal origin.[37,63,71] We thus hypothesized that locally released nucleotides within the bone microenviron-

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ment may act most effectively when potentiating the activity of systemic factors, and that this potentiation may provide a mechanism to localize systemic responses. This would seem particularly relevant during the turnover of the skeleton, primarily because bone remodeling is a focal process.[54]

To test this hypothesis, we investigated whether nucleotides could modulate the signaling activity of parathyroid hormone (PTH). PTH is the cardinal systemic regulator of bone and mineral homeostasis,[20,68] its most conspicuous action being regulation of calcium levels in extracellular fluid, primarily via actions on cells of the kidney and skeleton. The action of PTH on osteoblasts is complex and can result in the stimulation of both new bone formation and, paradoxically, resorption.[20,68] This ability of PTH to couple to opposing processes in remodeling has been attributed to the signaling properties of the PTH receptor (PTHR). This serpentine membrane receptor couples to Gq and Gs, and thereby initiates both calcium-driven and cyclic adenosine monophosphate (cAMP)-driven responses thought to be central to remodeling.[1] Many reports have linked PTH-induced cAMP accumulation to activation of downstream responses in osteoblasts; however, Gq activation and resultant calcium signaling in response to PTH remains somewhat controversial.[23,24,69]

There have been some reports that PTH stimulates small calcium elevations in osteoblasts.[23,43] We have been unable to reproduce this observation in settled clonal osteoblasts of rat and human origin or primary human osteoblasts in the presence of apyrase, suggesting that previously observed PTH-induced $[Ca^{2+}]_i$ mobilization may have arisen from nucleotide release resulting from shearing of stirred cell suspensions. Although PTHR1 activation by PTH(1-34) did not elevate $[Ca^{2+}]_i$ in rat UMR-106 osteosarcoma cells,[15] it led to a large increase in $[Ca^{2+}]_i$ when combined with activation of Gq-coupled $P2Y_1$ receptors. Notably, this synergy was independent of Ca^{2+} influx. The same effect was seen with PTH(1-31), which activates only Gs,[3] but not with PTH(3-34), which is reported to activate Gq.[27] Despite the link to Gs, this synergy does not involve cAMP accumulation or PKA activation, because forskolin (a potent activator of adenylyl cyclase) could not mimic synergy in the presence of ATP, and H89 (a potent PKA inhibitor) could not reduce the PTH- and ATP-induced $[Ca^{2+}]_i$ response. These findings are consistent with a study by Short et al. in which PTH-induced $[Ca^{2+}]_i$ mobilization was enhanced by agonists, including ATP, that acted at Gq-coupled receptors in PTHR1-transfected HEK-293 cells.[65] The mechanisms driving this potentiation are unclear and may differ between cell types. Other investigators have suggested that PTH may regulate $[Ca^{2+}]_i$ mobilization by facilitating translocation of Ca^{2+} between discrete intracellular stores, thereby regulating the Ca^{2+} pool available to receptors linked to IP_3 formation.[65] Alternatively, a direct G-protein interaction may account for potentiated $[Ca^{2+}]_i$ release.[39] These data clearly demonstrate that there are mechanisms in place to initiate calcium store release in response to PTH, but only in the presence of a locally released costimulant, such as ATP, which acts at a Gq-coupled receptor (**Figure 2**).

The significance of this calcium potentiation can be seen in its effects on transcription factor activation and gene expression. The transcription factor, CREB, binds the CRE and activates transcription upon its phosphorylation on Ser-133 in response to a number of extracellular signals, including elevated $[Ca^{2+}]_i$. Phospho-CREB plays a key role in signaling-driven activation of the protooncogene c-*fos*, which in turn has been strongly implicated in driving osteoblast proliferation and differentiation.[30] In UMR-106 cells, PTH and ADP costimulation results in increased levels of phospho-CREB and a synergistic induction of the endogenous c-*fos* gene.[15] Accordingly, the same costimulation

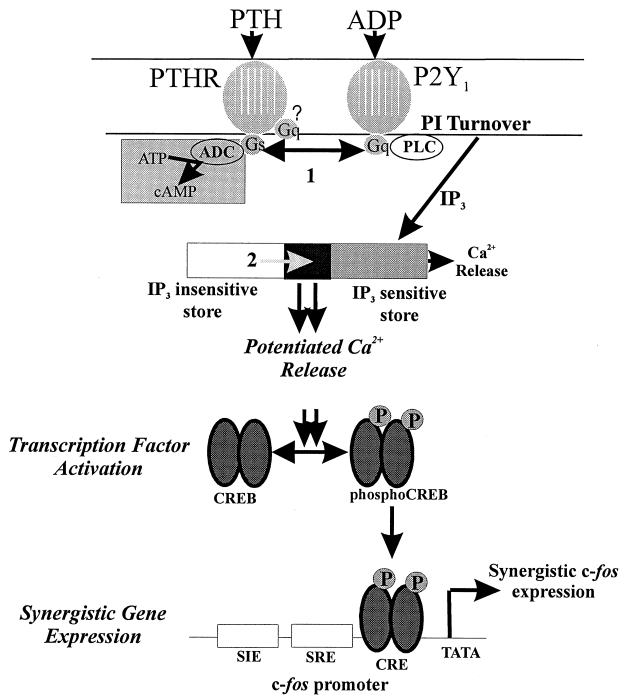

Figure 2. PTH potentiates nucleotide-induced signaling and gene expression in osteoblasts. PTH strongly potentiates nucleotide-induced calcium release from intracellular stores via a mechanism independent of PTHR1/Gq signaling. Despite the lack of requirement for cAMP accumulation or PKA activation, potentiated signaling is dependent on PTHR1 coupling to Gs. The mechanism driving this signaling synergy has been proposed to result from either: (1) a direct Gq/Gs protein interaction, or (2) shuttling of calcium from IP_3-insensitive to IP_3-sensitive stores. This potentiated calcium release results in increased levels of phospho-CREB and synergistic induction of the endogenous c-*fos* gene. This signaling cooperation between PTH and locally released, short-lived extracellular nucleotides represents a mechanism for initiating a highly targeted remodeling response.

synergistically activates a c-*fos* CaCRE-driven reporter gene in stably transfected UMR-106 cells. Interestingly, this contrasts with our previous study using the human osteosarcoma cell line, SaOS-2, which predominantly expresses $P2Y_1$ receptors, such as UMR-106 cells. In the SaOS-2 context, both Ca/CRE and the serum response element (SRE) of the c-*fos* promoter were required for synergy upon costimulation.[11] This coincided with the failure of PTH to potentiate nucleotide-induced $[Ca^{2+}]_i$ release in SaOS-2 cells.[11] Thus, multiple pathways, both calcium-dependent and -independent, couple $P2Y_1$ and PTH receptor coactivation to synergistic induction of gene expression in osteoblasts.

The ability of PTH to potentiate nucleotide responses is not restricted to $P2Y_1$ receptor activation. Primary human osteoblasts, derived from explant cultures of bone, predominantly express $P2Y_2$ receptors. As noted earlier, c-*fos* mRNA is synergistically induced when PTH is combined with UTP and ATP.[11] These data are very important, because they demonstrate that this synergy is not simply present in immortalized osteoblastic cell lines, but also occurs in a physiologically relevant context.

Systemic PTH can initiate remodeling at specific foci, a paradoxical but nevertheless well-established phenomenon that has defied explanation. In this review, we have provided a mechanism for this that involves the localized release of nucleotides by both lytic and, more importantly, nonlytic means. This costimulation strongly affects osteoblastic cells through the ac-

tivation of calcium-dependent intracellular signaling systems that ultimately target transcription factors and thereby synergistically induce the expression of genes necessary for osteoblast proliferation and differentiation.

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