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Molecular to Pharmacologic Control of Osteoblast Proliferation and Differentiation

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Abstract Control of osteoblast growth and development can be characterized from receptor mediated events to nuclear messengers controlling gene transcription. From this analysis it is possible to formulate a model to explain the reciprocal relationship between growth and differentiation as well as differential cytokine modulation of osteoblast function. Central to this model are putative tissue specific transcriptional switches (possibly of the bHLH gene superfamily) that may repress proliferation and permit the regulation of mature osteoblast phenotypic characteristics. This model proposes that in post-mitotic differentiated osteoblasts, tissue specific transcription factors determine the capacity to express osteoblastic characteristic, whereas receptor activated signalling cascades, namely, cAMP/protein kinase A, receptor serine/threonine kinase, and vitamin D receptor-dependent pathways, regulate mature osteoblast-specific gene expression. Activated differentiation switches also may feedback to transcriptionally repress proliferation. Conversely, in preosteoblasts, in which differentiation switches are turned off, distinct signalling cascades involving tyrosine kinases, PKC, and calcium/calmodulin regulate proliferation. Proliferating preosteoblasts also exhibit negative modulation of maturation either through inactivation of putative tissue-specific transcription factors and/or through AP-1 dependent phenotype suppression of genes expressed in mature osteoblast. Thus, the final outcome of transcriptional regulation of osteoblast function results from complex interactions between signalling pathways and permissive differentiating transcription factors. Though many aspects of this model remain speculative and require confirmation, it serves as a useful conceptual framework to further investigate the differential control of osteoblast proliferation and differentiation that may lead to improved pharmacologic ways to manipulate bone formation in vivo.

Key words: osteoblast differentiation, gene regulation, signal transduction pathways, osteocalcin, bHLH proteins

Pluripotent mesenchymal stem cells residing in the bone marrow stroma give rise to osteoblastic precursors that are committed to the osteoblast lineage and undergo a progressive and temporal expression of programmed development leading to mature functioning osteoblasts. Mature osteoblasts form a mineralized extracellular bone matrix, maintain autocrine control of osteoblastic growth and development, and regulate osteoclast recruitment and function (Fig. 1A) [Rodan, 1992]. All bone formation involves this developmental program, but the type of bone formed depends on the degree of skeletal maturation and the stimuli for bone formation. Bone formation either occurs as a process coupled to bone resorption (e.g., bone remodelling in the adult and endochondral bone formation during embryogenesis and the growing skeleton) (Fig. 1A) or de novo within the marrow cavity independent of bone resorption (e.g., neoosteogenesis in the adult or membranous ossification during embryogenesis) (Fig. 1B).

We are beginning to understand the factors that control the developmental pathway from mesenchymal commitment and proliferation to post-mitotic terminally differentiated osteoblasts. Regulation of the ordered osteoblastic developmental program involves 1) differential control of pre-osteoblast proliferation and mature osteoblast-specific gene expression via different growth factor/cytokines and separate intracellular signalling cascades, and 2) putative tissue-specific transcriptional switches that turn off proliferation and turn on the capacity to transcribe osteoblast specific genes. In this prospect, we will review the multitude of systemic

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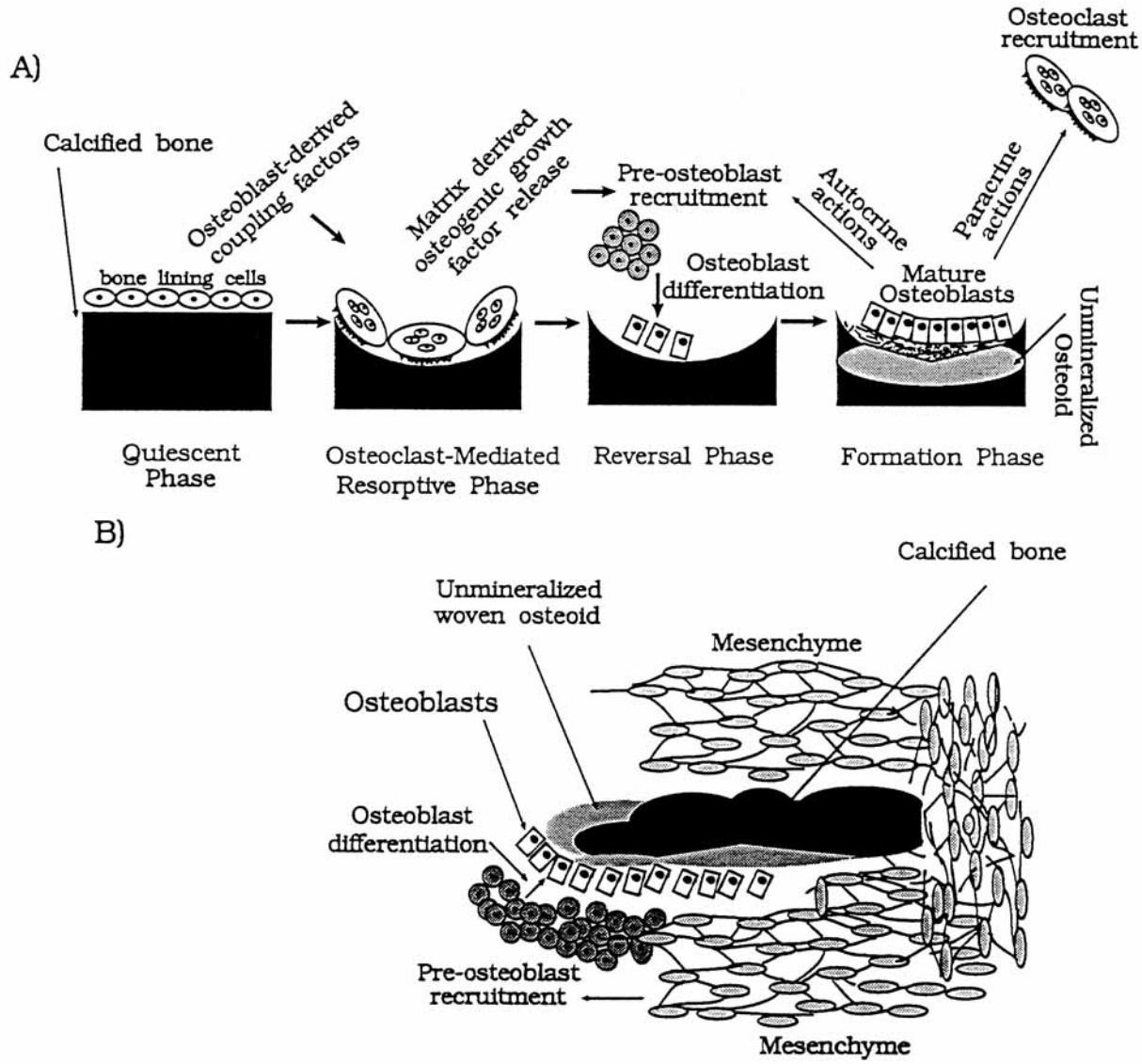

Fig. 1. Types of bone formation. **A:** Coupled bone formation. Coupled bone formation plays a physiologic role and occurs in geographically discrete packets on existing bone surfaces at sites of prior osteoclastic-mediated bone resorption. Endochondral bone formation is a similar sequential process that first involves cartilage formation that is followed by osteoclastic resorption of cartilage and its replacement by osteoblast-mediated bone formation. **B:** De novo bone formation. De novo bone formation (in the adult) and membranous ossification (during embryogenesis) arise directly from the expansion and differentiation of mesenchymal precursors to functioning mature osteoblasts that produce new trabecular structures within the marrow cavity. De novo bone formation in the mature skeleton is restricted to pathologic conditions and more recently to a variety of pharmacologic stimuli.

and local factors which modulate osteoblastic growth and development as well as characterize the various signal transduction pathways leading to differential control of proliferation and differentiation. In addition, we will examine models of transcriptional regulation that may explain the reciprocal relationship between growth and development in osteoblasts.

GROWTH FACTOR/CYTOKINE CONTROL OF OSTEOBLASTIC PROLIFERATION AND GENE EXPRESSION

Factors that stimulate growth have little effect on mature osteoblast gene expression, whereas differentiating factors often repress growth of replicating preosteoblasts. Such differ-

ential control appears to be mediated at both the receptor and signal transduction level (Fig. 2).

RECEPTOR TYROSINE KINASE (RTK) AGONISTS

In general, receptor tyrosine kinase (RTK) agonists play an important role in regulating osteoblast proliferation but have little effect on osteoblast differentiation in vitro. RTK subclasses share a common signal transduction pathway that is initiated by tyrosine phosphorylation of a homologous cytoplasmic domain in response to ligand binding [White, 1991; Pazin et al., 1992] (Fig. 2). A complex signaling kinase cascade follows that leads to transcriptional upregulation of cell proliferation. Though many details remain to be defined, activation of RTK relay their signal to the GTP binding protein, Ras, through two intermediary proteins, GRB2 and mSOS [Marx, 1993]. Ras, in turn, is linked to a kinase cascade that sequentially envolves raf-1, MEK, and mitogen-activated protein (MAP) kinases. This kinase cascade leads to phosphorylation of nuclear regulatory factors such as *myc* and *jun,* which are involved in transcriptional control of preosteoblastic proliferation (vide infra). RTK activation also increases the transcription of *c-fos* and other early response genes via the serum response element (SRE) in their promoter. These nuclear regulatory factors belong to the family of bZip proteins that form heterodimers that bind to AP-1 cis-acting domains in the promoter regions of proliferation associated genes. Other nuclear factors, such as the ets-family of transcription regulators [Reddy, 1992] may also be involved in transcriptional control of proliferation by the RTK/Ras pathway. In contrast, there are no known trans- or cis-acting activities that directly link RTKs to the transcriptional regulation of maturation-associated osteoblast genes such as alkaline phosphatase and osteocalcin. RTK-mediated increases in AP-1 activity, however, may repress osteoblast-specific gene transcription (vide infra).

Of the RTK agonists, IGF-I and -II are important systemic and local regulators of osteoblast function as documented by both in vivo and in vitro studies. IGFs transduce their signals through the heterotetrameric class II RTK. The IGFs, which are made by osteoblasts and stored in bone matrix, can act as either autocrine or hormonal factors to regulate bone remodelling [Canalis, 1993]. Osteoblast production of IGF-I is responsive to hormonal regulation by parathyroid hormone, estrogen, thyroxine, growth hormone, and glucocorticoids, suggesting that IGF-I may mediate the effects of these systemic hormones on bone. In addition, a multitude of circulating as well as locally produced IGF binding proteins (IGF-BPs) modulate the actions of the IGFs by serving as a reservoir to prolong the metabolic half-life of IGFs, or by buffering the activity of circulating IGF, thereby reducing IGF bioactivity [Mohan et al., 1989, Chen et al., 1991a]. The overall effect of IGF-I when administered systemically to animals is to stimulate coupled bone formation [Degerblad et al., 1992]. A concordant increase in osteoclast mediated bone resorption limits the increase in bone density following systemic administration of IGF-I [van der Veen and Netelenbos, 1990]. IGF-I does not induce de novo bone formation. In vitro, IGFs enhance bone cell replication and stimulate pro-α_1(I)-collagen mRNA expression [Schmid et al., 1989] but do not increase alkaline phosphatase or osteocalcin, two markers of osteoblast differentiation [Ohta et al., 1992].

EGF and PDGF, which act through class I and III RTKs, respectively, stimulate osteoblast proliferation in vitro, but their role in vivo is less clear. Cross-talk between other signalling pathways distinguishes these RTK agonists from IGF-I. EGF and PDGF activate PLCγ leading to stimulation of phosphatidyl inositol bis-phosphate hydrolysis and the release of two key second messengers inositol tri-phosphate (IP_3) and diacylglycerol (DAG). The subsequent increase in intracellular calcium and PKC activation may have different effects on osteoblast function than the tyrosine kinase dependent cascades (vide infra). In contrast, the RTK for IGF-I does not activate calcium and PKC dependent pathways in osteoblasts [Quarles et al., 1993].

RECEPTOR SERINE/THREONINE KINASES

Protein extracts from demineralized bone are capable of stimulating endochondral and de novo bone formation when injected into nonosseous tissues [Urist, 1965]. Recently, bone morphogenic proteins have been cloned and structurally characterized [Wozney et al., 1988]. Bone morphogenic proteins (BMP) 2–7, are members of the TGF-β supergene family, whereas BMP-1 appears to be unrelated to other BMPs and may act as a binding protein and/or co-activator [Zheng et al., 1992]. BMP-7 is also referred to as osteogenic protein (OP-1) [Sampath et al., 1992].

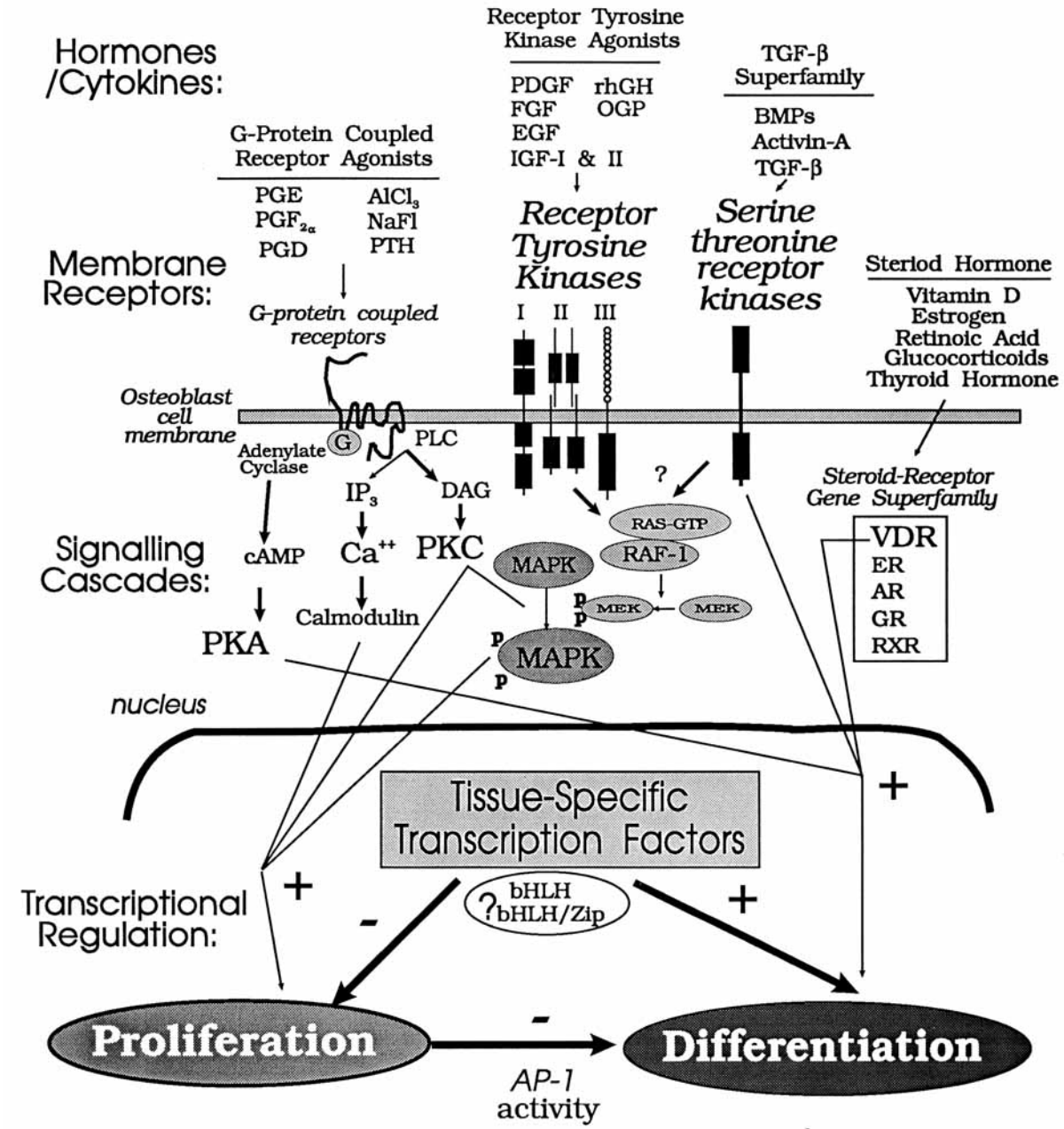

Fig. 2. Model of osteoblast growth and development. Central to the differential regulation of osteoblast growth and development are putative tissue-specific transcription factors that may act as a nodal point for the transition between proliferation and differentiation. These transcription switches that turn on osteoblast differentiation and turn off proliferation have not been defined in osteoblasts, though recent data suggest that they may be members of the HLH family of nuclear regulatory factors. We postulate that HLH heterodimers repress transcription of early response genes, such as c-fos, and upregulate osteoblast specific genes, such as osteocalcin. Superimposed upon these differentiation switches are additional regulator elements that modulate the intensity of proliferation and differentiation. Stimulation of membrane receptors (e.g., receptor tyrosine kinases, receptor serine/threonine kinases, and G-protein coupled receptors) as well as cytosolic receptors (e.g., vitamin D, retinoic acid, and estrogen receptors) lead to stimulation of separate signalling cascades which couple early biochemical events from divergent pathways to transcriptional activities required for proliferation and differentiation. For instance, proliferation is repressed by cAMP and vitamin D dependent pathways. Proliferation is induced by tyrosine kinase and PKC-dependent pathways. Conversely, the osteoblast phenotype is upregulated by cAMP, vitamin D, and receptor serine/threonine dependent pathways and repressed by increased AP-1 activity that occurs in proliferating cells. Although this scheme is an over-simplification, and parts of it remain speculative, it serves as a working model to examine the mechanism(s) whereby growth factors and cytokines differentially induce replication of committed preosteoblast stem cells and modulate their subsequent differentiation to functionally mature osteoblasts.

TGF-β and activin are related proteins in this superfamily that also affect osteoblast growth and development. TGF-β, activin, and BMPs are ligands for membrane receptors associated with a serine/threonine-specific protein kinase activity [Lin et al., 1992].

Two subclasses of receptor serine/threonine kinases, a 55 kDa (Type I) and a 70 kDa (Type II) receptor, have been identified (Fig. 2). The signalling pathways involved in the actions of these receptors are not well studied, but retinoblastoma repressor gene product (Rb) may be central to growth repression by these maturation and osteoinductive factors. Most of the available information is derived from the actions of TGF-β in nonosteoblast model systems. These studies suggest that Type II receptors possibly in conjunction with Type I receptors inhibit growth by mechanisms related to hypophosphorylation of the retinoblastoma gene product (Rb), which is involved in control of cell cycle progression [Whitson and Itakura, 1992]. TGF-β1 also inhibits c-myc transcription through the retinoblastoma repressor gene product [Munger et al., 1992]. The fact that mutations in the retinoblastoma gene are involved in the pathogenesis of osteosarcomas and other bone tumors, which are characterized by abnormal regulated growth [Araki et al., 1991] supports the role of Rb in osteoblast growth control.

This superfamily of osteoinductive proteins act as local rather than systemic factors. Most studies have evaluated their potential to induce ectopic bone formation by administering the BMPs subcutaneously with a collagen matrix carrier. The collagen matrix induces attachment and proliferation of mesenchymal cells which in response to the BMPs differentiate to form new bone. BMP2 and BMP7 (hOP-1) are capable of inducing endochondral and membranous bone formation under these experimental conditions, whereas activin and TGF-β (which shares only a 34% homology with hOP-1) does not induce ectopic bone formation [Hammonds et al., 1991]. TGF-β stimulates bone formation, however, when applied directly to the periosteum of bone [Marcelli et al., 1990] and facilitates the repair of bone [Noda and Camilliere, 1991]. The overall effects of BMPs in vitro are to stimulate differentiation of uncommitted mesenchymal cells into the chondrocyte and osteoblast lineage and to repress proliferation [Vukicevic et al., 1991; Thies et al., 1992]. The different BMPs, however, exhibit variable effects on individual components of mature osteoblast function. BMP-4 and BMP-7 stimulate, whereas BMP-2 has no effect on Type I collagen synthesis [Chen et al., 1991b]. Most BMPs upregulate markers of mature osteoblasts such as alkaline phosphatase and osteocalcin expression. The mechanisms underlying these different responses to BMP subclasses remains to be defined.

Though activin-A does not stimulate de novo bone formation in vivo, recombinant human activin-A binds to putative membrane receptors in osteoblasts, stimulates DNA and collagen synthesis, and inhibits alkaline phosphatase in various osteoblast cultures [Hashimoto et al., 1992; Centrella et al., 1991]. The in vitro actions of TGF-β in osteoblasts are complex and opposing results have been obtained from different model systems [Zheng et al., 1992]. Most studies suggest that TGF-β either inhibits or stimulates replication of osteoblasts but does not upregulate osteoblast related gene expression including alkaline phosphatase and osteocalcin [Chen et al., 1991b; Ohta et al., 1992].

G-PROTEIN COUPLED RECEPTOR AGONISTS

Guanine nucleotide binding protein (G-protein) coupled receptors are characterized by a ligand binding C-terminus, conserved 7 hydrophobic transmembrane domains, and an intracellular N terminus that contains binding sites for a group of heterotrimeric G-proteins [Birnbaumer et al., 1990]. Different isoforms of Gα subunits are linked to different receptor systems and signal transduction cascades [Olate and Allende, 1991]. The work that directly characterizes G-protein receptor structure and function has been performed in nonosteoblast cell systems and is beyond the scope of this review. Information regarding the presence and function of this G-protein receptor class in osteoblasts is mostly inferred from ligand binding and activation of the various signal transduction cascades known to be associated with G-protein receptor activation.

G-proteins activated either through specific receptors (such as those for PTH and prostaglandins) or in response to fluoride and aluminum, stimulate several major signaling cascades in osteoblasts (Fig. 2) [Hakeda et al., 1987]. One pathway involves phospholipase C (PLC) and/or PLD mediated phosphoinositide and phosphatidylcholine turnover, which results in generation of IP_3 and diacylgycerol (DAG) second messengers. IP_3 stimulates release of intracellular cal-

cium and activation of the calcium/calmodulin signaling cascade, whereas DAG stimulates protein kinase C, which activates serine/threonine phosphorylation cascades [Nishizuka, 1992]. The other major pathway involves adenylate cyclase generation of cAMP and consequent activation of PKA dependent protein kinase cascades. Cross-talk exists within these signaling cascades activated by G-proteins as well as between those stimulated by RTK and steroid receptors. For instance, cAMP can modulate calcium influx [Yamaguchi et al., 1988] and mediate the heterologous upregulation of the VDR [van Leeuwen et al., 1992a]. PKC and calcium may exert feedback activation of phospholipase D resulting hydrolysis of phosphotidylcholine to form DAG and phosphatidic acid [Exton, 1990]. PKC can also interact downstream with the RTK cascade by stimulating putative non-receptor tyrosine kinases [Quarles et al., 1993]. This accessory PKC pathway is involved in mediating the proliferative response to $PGF_{2\alpha}$ in MC3T3-E1 osteoblasts. Recent studies suggest that PKC may enter this pathway through Raf-1 kinase and down stream activation of MAP kinases [Marx, 1993].

These G-protein coupled signalling cascades have different effects on proliferation and differentiation in osteoblasts. Activation of the calcium/calmodulin dependent and the DAG/PKC cascades stimulate proliferation. Similar to RTKs and other mitogenic signals, PKC-mediated transcriptional activation of proliferation is mediated, in part, through AP-1 activity of bZip early response genes. The transcriptional control by the calcium/calmodulin pathway is less well defined. Recent studies demonstrating that the retinoblastoma gene product (Rb) phosphorylation is stimulated by the calcium/calmodulin pathway suggest that this pathway may be involved [Takuwa et al., 1992]. In contrast, cAMP/PKA cascade is antiproliferative and results in upregulation of certain osteoblast phenotypic characteristics, such as alkaline phosphatase activity and collagen production [Hakeda et al., 1987]. cAMP/PKA signaling pathways regulate gene transcription through CREB/ATF proteins. CREB/ATF proteins, like fos and jun, belong to the superfamily of bZip proteins, but unlike AP-1 proteins, CREB/ATF are constitutively expressed and relatively unresponsive to extracellular stimuli. Transcriptional activity of CREB by cAMP is controlled post-translationally by PKA mediated phosphorylation [Hoeffler, 1992]. cAMP response elements have been found in the promoter regions of both alkaline phosphatase and osteocalcin genes which are early and late markers, respectively, of the mature osteoblast phenotype (Fig. 3).

There are multiple factors affecting osteoblastic growth and differentiation that act through G-protein coupled receptors. PTH has a well known physiologic role in bone metabolism, being important in regulating calcium and phosphorus homeostasis. PTH is a 84 amino acid protein secreted into the blood by the parathyroid gland in response to various physiologic and pathologic conditions [Kronenberg, 1993]. PTH stimulates both cAMP/PKA and PLC/PLD dependent signalling cascades in osteoblasts. With regard to its osseous effects in both humans and animals, PTH typically stimulates coupled bone formation, though at pharmacologic levels de novo bone formation can occur within the marrow cavity. The osseous effects of PTH are mediated by direct actions on osteoblasts as well as by secondary effects mediated by PTH stimulates production of 1,25 $(OH)_2$ vitamin D by the kidney. The in vitro effects of PTH are paradoxical to its in vivo actions. Whereas PTH simulated osteoblast recruitment and bone formation in vivo, PTH suppresses replication and differentiation of osteoprogenitor cells in culture [Bellows et al., 1990]. Other studies show that PTH stimulates production of IGF-I by osteoblasts in culture [McCarthy et al., 1989]. Such autocrine effects of PTH may explain its anabolic effects on bone formation in vivo.

Prostaglandins are eicosinoids derived from arachidonate metabolism that are locally produced by osteoblasts and possibly other cells in bone marrow. Membrane receptors for prostaglandins are likely coupled to G-proteins [Quarles et al., 1993]. These factors also stimulate both cAMP and PLC dependent pathways. Prostaglandins of the PGE series predominately stimulate cAMP and upregulate differentiated function, whereas $PGF_{2\alpha}$ stimulates PLC dependent pathways and proliferation in vitro [Yamaguchi et al., 1988]. $PGF_{2\alpha}$ also stimulates putative down-stream nonreceptor tyrosine kinases via PKC activation in osteoblasts [Quarles et al., 1993]. Eicosinoids play an autocrine and paracrine role in normal bone metabolism, but their local and systemic administration to animals is capable of stimulating coupled bone formation and possibly de novo bone formation [Jee et al., 1992; High, 1988]. PGE_2 also plays a

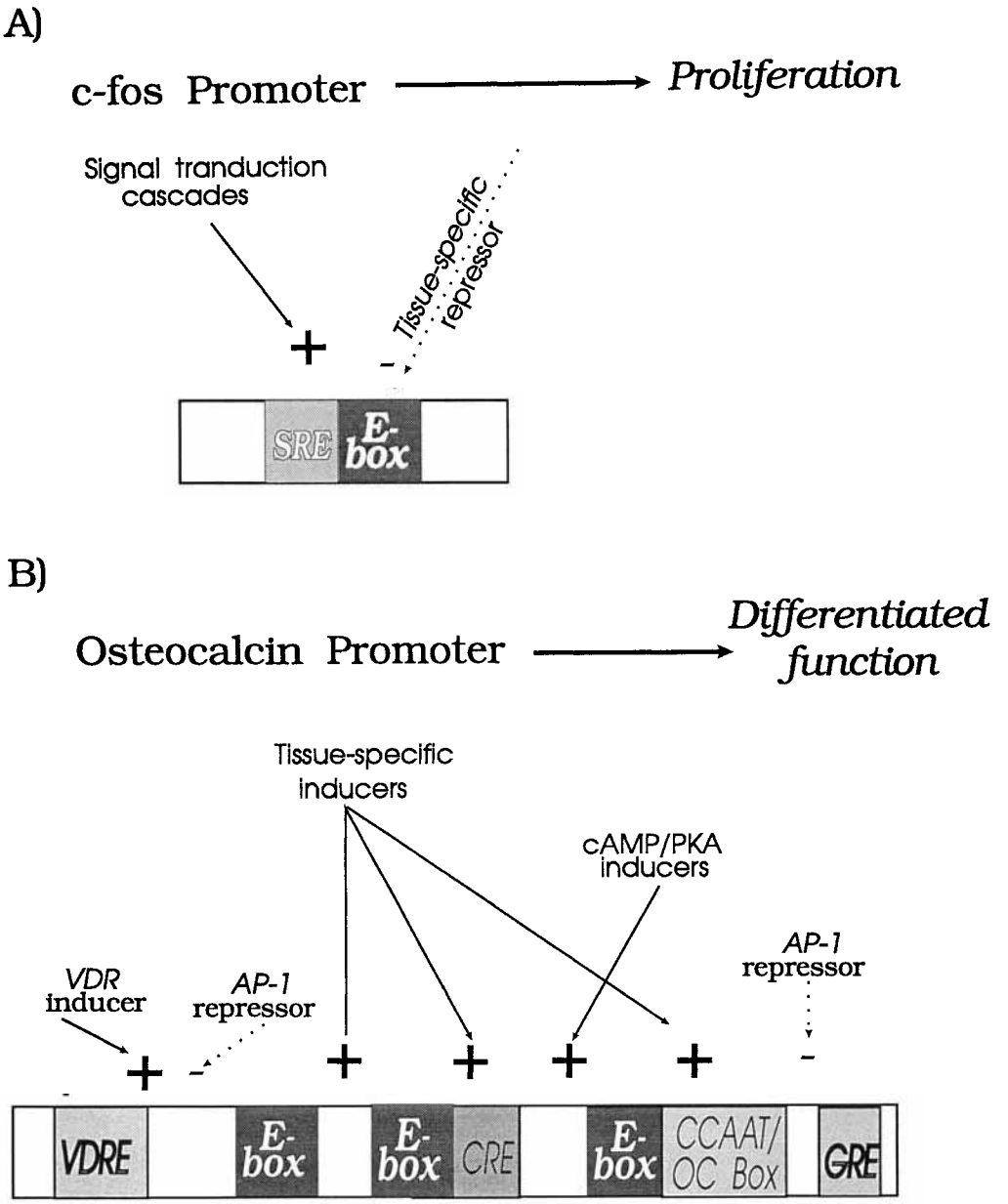

Fig. 3. Cis-acting DNA binding domains in the promoter region of the c-fos and osteocalcin gene. **A:** The c-fos promoter contains binding domains for the serum response element (SRE), which transduces signals from multiple mitogenic pathways including receptor tyrosine kinases and G-protein coupled receptors agonists. An E-box binding motif overlaps the SRE and may function to repress c-fos expression in differentiated cells. **B:** CANNTG E-box enhancers are also present in the promoter regions of osteoblast-related genes including alkaline phosphatase, osteopontin, and osteocalcin. Shown is a representation of the osteocalcin promoter. Three E-boxes (one CACATG and two CAGCTG) are located within 300 bp of the transcription start site. There is overlap of the CACATG and the osteocalcin (OC) box, which is involved in basal expression of osteocalcin. There is also overlap between an E-box and cAMP response element (CRE). The function of these E-box binding sites in controlling osteocalcin expression remain to be defined. Coordinate occupancy of vitamin D response element (VDRE) and OC box by AP-1 may repress expression of osteocalcin.

central role in the local regulation of bone resorption [Harvey and Bennett, 1988].

Fluoride is also able to stimulate bone formation that is coupled to osteoclastic mediated bone resorption [Kellner, 1939; Snow and Anderson, 1986]. In humans and various animal models fluoride increases osteoblast number and matrix synthesis as well as osteoclastic activity. The osteoinductive activity of fluoride generally predominate leading to increased cancellous bone

mass. Fluoride has variable effects on osteoblasts in vitro that depends on the culture model and possibly the state of maturation. Fluoride stimulates bone nodule formation and alkaline phosphatase activity in osteoblast progenitors, whereas it has no effect on mature human osteoblasts in culture. Other studies suggest that fluoride effects on osteoblasts are mediated by enhancing the activity of bone cell mitogens [Farley et al., 1990].

In a series of recent investigations we found that aluminum administration to dogs results in de novo bone formation [Quarles et al., 1988]. Aluminum stimulation induces a unique osteogenic response within the marrow cavity which results in formation of new structural bone (trabecular) units independent of preceding bone resorptive activity, a process that we have called neo-osteogenesis (Fig. 1B). Its actions are limited to the marrow cavity where mesenchymal precursors may be responsible for mediating the formation of woven bone. In vitro, aluminum is a potent stimulant of DNA synthesis and *c-fos* gene expression in immature osteoblasts [Quarles et al., 1991]. Aluminum appears to be acting via PKC dependent pathways possibly through activation of G-proteins. Additional studies suggest that aluminum stimulates collagen synthesis and enhances calcitriol mediated increases in osteocalcin. These actions appear to be distinct from the in vitro actions of fluoride [Lau et al., 1991].

STEROID HORMONE RECEPTOR SUPERFAMILY

Steroid hormones, which are small hydrophobic molecules synthesized from cholesterol, control a variety of physiologic processes [Burnstein et al., 1993]. The presence of receptors for a variety of steroid hormones in osteoblasts has been well documented (Fig. 2). These include receptors for vitamin D, estrogen, retinoic acid, glucocorticoids, and thyroid hormone [Canalis, 1993; Colvard et al., 1989]. These intracellular receptors act through a common mechanism of ligand dependent transcriptional modulation that begins with steroid binding to its receptor, translocation of the ligand/receptor complex to the nucleus, and interaction of this complex with cis-acting domains in promoter regions of various genes to regulate their transcription. The differential expression of receptor binding elements in gene promoters as well as modulation of receptor number and activity by other signalling pathways may account for the specific actions of various steroids in osteoblasts.

Vitamin D regulation of gene transcription is a paradigm for the steroid receptor superfamily in osteoblasts [Norman et al., 1993]. Most of the effects of vitamin D can be attributed to interaction with its cytosolic receptor which translocates to the nucleus and interacts with cis-acting vitamin D response element (VDRE) to positively regulate gene transcription. Two classes of VDRE have been defined. One confers response to vitamin D alone, whereas the other is activated by heterodimers between VDR and the retinoid-X receptor-alpha that act as co-regulators of gene transcription [Carlberg et al., 1993]. The latter may explain the synergistic interaction between the vitamin D and retinoic acid with regard to expression of osteocalcin [Oliva et al., 1993]. The VDRE appears to be located in all of the genes that are upregulated in mature osteoblasts. Vitamin D also has non-genomic functions in osteoblasts that lead to increased levels of intracellular calcium and stimulation of phospholipid metabolism [Baran et al., 1992; Haining et al., 1988].

Similar to receptor serine/threonine agonists and cAMP/PKA pathways (vide supra), vitamin D decreases proliferation and induces differentiation of osteoblasts in vitro. However, unlike the BMPs, vitamin D effects on differentiation involve upregulation of genes that are already expressed in the mature osteoblast, whereas BMPs appear to initiate the process of tissue specific gene expression [Owen et al., 1991; Ohta et al., 1992]. Vitamin D induces the synthesis of collagen, alkaline phosphatase, osteocalcin, and osteopontin in osteoblasts [Franceschi et al., 1988]. The in vitro effects of vitamin D may be altered by interaction with other signalling pathways either through VDR regulation or alterations in transcriptional activation. Agents such as parathyroid hormone, retinoic acid, estrogens, and vitamin D itself can upregulate the VDR [van Leeuwen et al., 1992b; Suzuki et al., 1993]. In addition, the cAMP signalling cascade, which also promotes differentiation, can upregulate the VDR in osteoblasts [van Leeuwen et al., 1992a]. Though VDR is selectively phosphorylated by PKC [Hsieh et al., 1991] it is not clear whether this is responsible for the PKC mediated downregulation of VDRE. On the other hand, EGF [van Leeuwen et al., 1991] and PKC [van Leeuwen et al., 1992b], which stimulate

pre-osteoblast proliferation, have been shown to decrease VDR number.

Increased AP-1 activity associated with proliferation may interfere with vitamin D actions to stimulate differentiation. AP-1 binding sites reside within the basal regulatory OC box and the vitamin D-response enhancer element (VDRE) (Fig. 3). There is mutually exclusive occupancy of the VDRE and AP-1 sites, such that enhanced AP-1 activity in proliferating osteoblasts repress vitamin D-mediated upregulation of osteocalcin [Owen et al., 1993]. Such phenotype suppression may partially explain the reciprocal relationship between proliferation and differentiation in osteoblasts.

Vitamin D supplementation does not lead to an increase in bone mass or a net increase in bone accretion but may play a permissive role in osteoblast-mediated bone formation in vivo. Glucocorticoids, on the other hand are associated with suppression of bone formation and osteoblast function as well as increased bone resorption in vivo. The overall effects of glucocorticoids are to reduce bone mass. Retinoic acid (vitamin A) administration can induce hypercalcemia in vivo due to its actions to increase bone resorption [Trechsel et al., 1987]. Retinoic acid appears to be involved in embryogenesis of limb bud development and endochondral ossification [Campbell and Kaplan, 1992], but little is known about its effects on bone formation in the adult skeleton. Estrogens appear to stimulate bone formation, though there is controversy regarding their osteoinductive actions [Turner, 1991].

DIFFERENTIAL CONTROL OF OSTEOBLAST GROWTH AND DEVELOPMENT: ROLE OF OSTEOBLAST-SPECIFIC SWITCHES

Growth factor/cytokine, receptors, and intracellular signals are insufficient to explain the regulation of osteoblast growth and development. The step-wise postproliferative expression of multiple osteoblastic features [Quarles et al., 1992] and the inability of pre-osteoblast to express an osteoblastic phenotype in response to pharmacologic stimuli suggest that additional factors may be involved in the tissue-specific regulation of osteoblastic maturation [Yohay and Quarles, 1992; Owen et al., 1991]. Indeed, the ability to express the osteoblast phenotype may be intrinsic to the maturation process and related to the factors that switch on the commitment toward the osteoblastic lineage. There is growing evidence that osteoblast-specific transcription factors may be involved in triggering osteoblast differentiation and inhibiting preosteoblast proliferation.

The family of helix-loop-helix (HLH) DNA-binding proteins, which are important for transcriptional regulation of cellular differentiation in a variety of tissues, are potential candidates for controlling osteoblastic development [Tapscott and Weintraub, 1991; Edmondson and Olson 1993]. bHLH transcription factors contain two conserved amphipathic alpha helixes joined by a nonconserved "loop" segment which serve as an interface for dimerization, which brings together the basic regions of HLH proteins to from a composite DNA-binding domain. Tissue-specific gene transcription is positively regulated by formation of heterodimers between these tissue-specific bHLH proteins and non-cell-specific bHLH regulatory elements such as E12/E47 and negatively regulated by binding to Id, a negative regulatory HLH protein lacking the basic DNA binding domain. These heterodimers interact with the E-box cis-acting elements (CANNTG) located in the promoter/enhancer region of tissue specific genes.

Though no osteoblast-specific HLH proteins have yet been identified, indirect evidence suggests that HLH proteins may be involved in upregulating differentiation and repressing proliferation in osteoblasts. With regard to differentiation, E-box binding domains are present in the osteocalcin, osteopontin, and alkaline phosphatase gene promoters, which are sequential markers of osteoblast differentiation (Fig. 3). In addition, there is functional data linking bHLH proteins to the expression of the osteoblastic phenotype through E-box binding motifs in the osteocalcin promoter. In this regard, Id mRNA, which inhibits bHLH actions, is abundantly expressed in proliferating and undifferentiated MC3T3-E1 preosteoblasts and decreases prior to osteoblast-specific gene expression [Ogata and Noda, 1991]. This inverse relationship between Id expression and osteoblastic differentiation is consistent with Id involvement in osteoblastic differentiation. Moreover, overexpression of Id prevents expression of alkaline phosphatase in developing osteoblasts [Murray et al., 1992]. In addition, various other non-tissue-specific regulatory bHLH proteins, including *twist* and E-proteins, have been identified in osteoblasts. Recent studies in malignant Ros 17/2.8 osteoblasts also support a role of HLH proteins in the control of osteoblastic-related gene expression.

In Ros 17/2.8 cells, vitamin D-mediated upregulation of osteocalcin is associated with decrements in Id and increased binding of nuclear extracts to E-box motifs [Kawaguchi et al., 1992].

bHLH proteins may also repress proliferation by inhibiting the transcription of early response genes. In this regard, E-box cis-acting domains overlap the serum response element in the c-fos promoter (Fig. 3). In non-osteoblastic systems, members of the bHLH family of proteins have been shown to act as negative regulators of c-fos transcription (and cellular proliferation) by blocking the transactivating function of the SRE [Trouche et al., 1993]. This provides a mechanism whereby tissue-specific transcription factors in osteoblasts can repress proliferation.

Though the presence of bHLH proteins in osteoblasts and their apparent regulation by factors modulating differentiation suggest that osteoblastic-specific HLH family members may control the osteoblastic developmental program, the evidence for a functional role in osteoblast is incomplete. Additional studies are needed to define specific bHLH protein in osteoblasts and characterize their role in the transcriptional control of osteoblast specific gene expression.

Understanding the molecular control of osteoblast function and its relationship to external stimuli and intracellular signalling systems is fundamental to knowing how these cells differentiate and proliferate. Such information may permit the development of pharmacologic approaches to selectively manipulate pre-osteoblast replication, the differentiation process and mature osteoblast function. The ability to manipulate these components of the osteoblast developmental sequence may allow separate induction of coupled and de novo bone formation, which would be useful for treatment of a variety of human disease states characterized by osteopenia or overabundant osseous production.

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