

# THE DEVELOPMENTAL CONTROL OF OSTEOBLAST-SPECIFIC GENE EXPRESSION: ROLE OF SPECIFIC TRANSCRIPTION FACTORS AND THE EXTRACELLULAR MATRIX ENVIRONMENT

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**ABSTRACT:** Bone formation is a carefully controlled developmental process involving morphogen-mediated patterning signals that define areas of initial mesenchyme condensation followed by induction of cell-specific differentiation programs to produce chondrocytes and osteoblasts. Positional information is conveyed *via* gradients of molecules, such as *Sonic Hedgehog* that are released from cells within a particular morphogenic field together with region-specific patterns of hox gene expression. These, in turn, regulate the localized production of bone morphogenetic proteins and related molecules which initiate chondrocyte- and osteoblast-specific differentiation programs. Differentiation requires the initial commitment of mesenchymal stem cells to a given lineage, followed by induction of tissue-specific patterns of gene expression. Considerable information about the control of osteoblast-specific gene expression has come from analysis of the promoter regions of genes encoding proteins like osteocalcin that are selectively expressed in bone. Both general and tissue-specific transcription factors control this promoter. *Osf2/Cbfa1*, the first osteoblast-specific transcription factor to be identified, is expressed early in the osteoblast lineage and interacts with specific DNA sequences in the osteocalcin promoter essential for its selective expression in osteoblasts. The *OSF2/CBFA1* gene is necessary for the development of mineralized tissues, and its mutation causes the human disease, cleidocranial dysplasia. Committed osteoprogenitor cells already expressing *Osf2/Cbfa1* must synthesize a collagenous ECM before they will differentiate. A cell:ECM interaction mediated by integrin-type cell-surface receptors is essential for the induction of osteocalcin and other osteoblast-related proteins. This interaction stimulates the binding of *Osf2/Cbfa1* to the osteocalcin promoter through an as-yet-undefined mechanism.

**Key words.** Bone formation, gene expression, transcription.

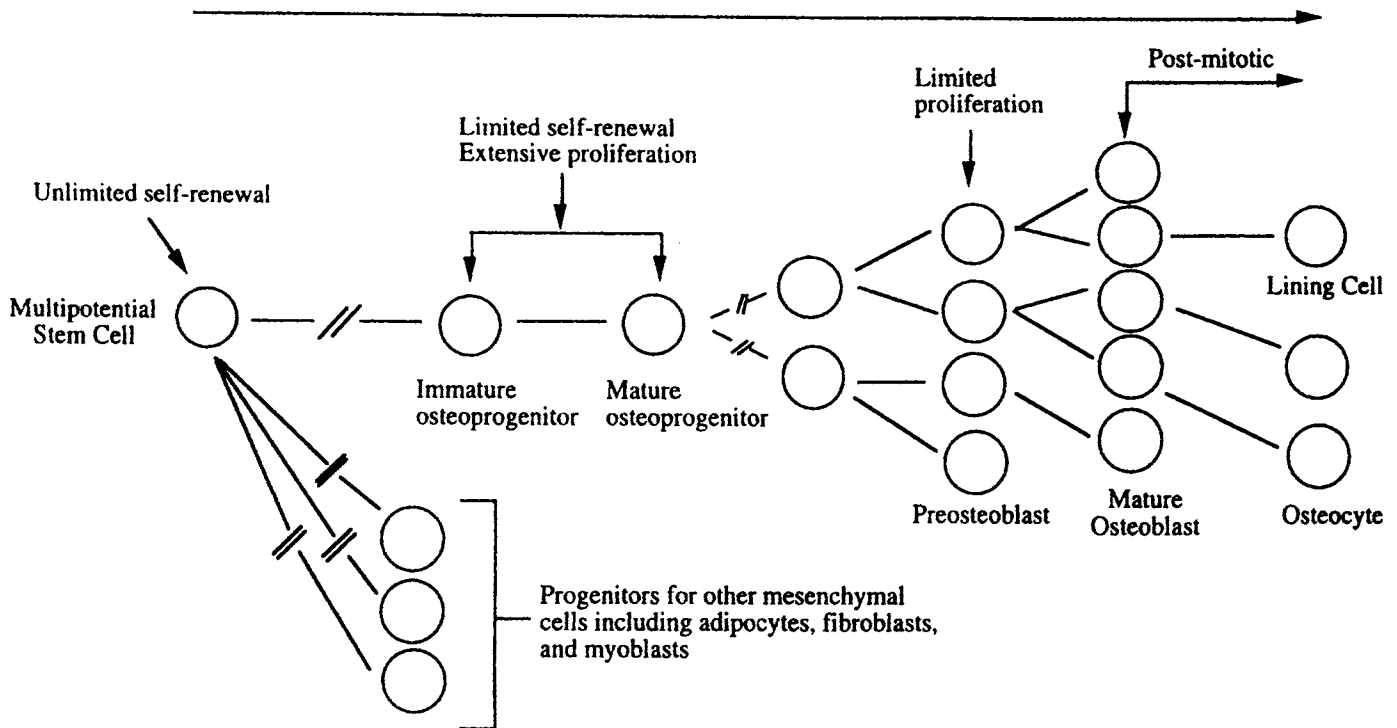
## Introduction

An understanding of factors controlling gene expression in osteoblasts is of particular importance to dental researchers, both because of the fundamental role played by bone in the overall structure of oral tissues and because underlying principles involved in the control of osteoblasts will likely be relevant to other mineralized tissues such as dentin and cementum. The goal of this article is to provide a timely overview of current knowledge regarding early events in bone formation, with emphasis on the nuclear factors required for the selective expression of specific genes in osteoblasts and their regulation by extracellular matrix signals.

## The Embryonic Patterning of Bone

There are two broad phases of tissue morphogenesis; an initial patterning phase, in which cells destined to form a given tissue are specified in time and space, and the subsequent induction of the necessary cellular phenotypes to produce the tissue of interest. These phases are not mutually exclusive. Rather, cells within a developing tissue can be viewed as altering the pattern of differentiation of their neighbors through a variety of signals mediated by diffusible factors, cell:extracellular matrix interactions, and direct cell:cell contact.

The patterning of bones and teeth has been the subject of several recent reviews and will not be extensively discussed in this article (Erlebacher *et al.*, 1995; Thesleff

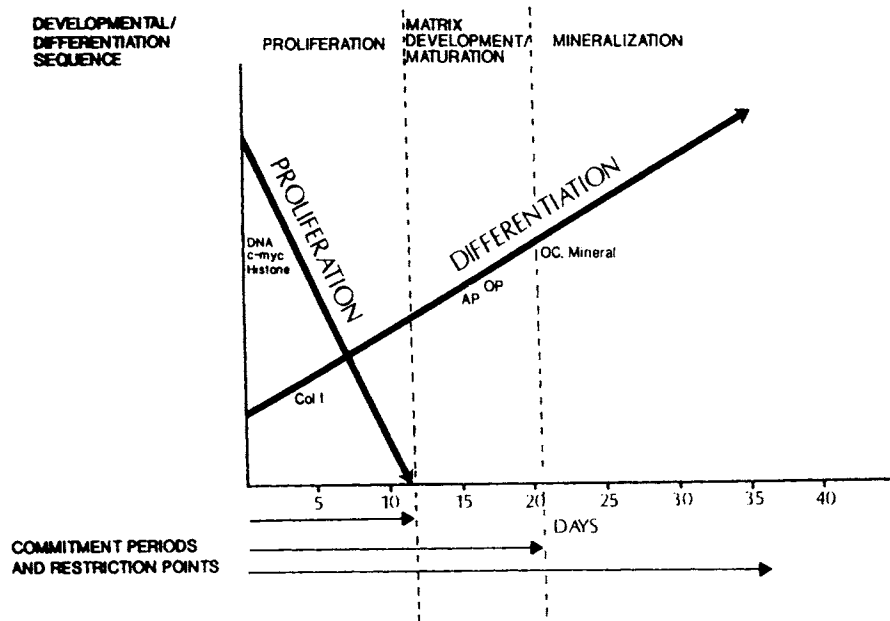


**Figure 1.** Proposed steps in the osteoblast lineage implying recognizable stages of differentiation. All mesenchymal lineages are postulated to be derived from a common stem cell precursor (adapted from Aubin and Lui, 1996).

*et al.*, 1995; Johnson and Tabin, 1997). However, a few common themes will be briefly outlined. The developing limb bud serves as a good example of the types of interactions necessary for skeletal morphogenesis (Tickle, 1995). Early in limb development, an anterior/posterior axis is established which specifies the positions of bones such as the digits of the hand. This axis is determined by a group of cells in the posterior limb mesenchyme known as the Zone of Polarizing Activity (ZPA). These cells were identified by their ability, when transplanted to the anterior side of the limb bud, to induce a mirror-image duplication of skeletal elements. *Sonic Hedgehog* (Shh), a secreted protein, is the key inductive factor produced by the ZPA. The anterior/posterior asymmetry of limb bones is thought to be a reflection of the distribution of Shh throughout the limb bud as it is secreted from the ZPA. The primary targets of Shh are cells of the apical ectodermal ridge (AER) which, after Shh stimulation, produce Fibroblast Growth Factor 4 (FGF-4). FGF-4 in turn stimulates Shh secretion in the mesenchyme underlying the AER. Shh also stimulates the homeobox gene, *hoxd-13*, which is required for normal limb patterning, and Bone Morphogenetic Protein 2 (BMP2), an inducer of cartilage condensation and subsequent bone formation. Thus, early limb morphogenesis is explained by a reciprocal pattern of inductive signals between underlying

limb mesoderm and epithelium (AER) initiated by the asymmetric distribution of a signal originating from the ZPA. The overall result of these interactions is specification of the positional identity of skeletal elements well before the initiation of overt skeletogenesis. Recent evidence suggests that similar patterning events may take place in craniofacial bone formation and tooth morphogenesis (Satokata and Maas, 1994).

The initial positional identity of skeletal precursors is defined by the pattern of expression of a family of transcription factors encoded by homeobox genes (reviewed in Erlebacher *et al.*, 1995). Consistent with the concept that they provide positional information, targeted inactivation, mutations, or ectopic expression of homeobox genes leads to deletion or addition of specific skeletal structures. For example, inactivation of the *hoxa-2* gene results in deletion of the craniofacial elements derived from the second branchial arch and their replacement by elements derived from the first branchial arch (Gendron-Maguire *et al.*, 1993). The homeotic mutation does not, therefore, affect bone formation *per se*. Rather, it alters the type of bony structure to be formed at a given position. The initial pattern defined by the expression of homeobox genes is further modified by locally secreted factors such as BMPs. Certain homeotic genes, including members of the *msx* and *dlx* families, may also have direct



**Figure 2.** Relationship between cell growth and differentiation-related gene expression during the *in vitro* cultivation of calvarial osteoblasts. The reduction in cell growth and concomitant increase in differentiation are indicated by arrows. H4 histone gene expression is most closely correlated with cell division. Other genes expressed at this time are *c-fos* and *c-jun* (see text). Abbreviations: Col 1, type I collagen; AP, alkaline phosphatase; Op, osteopontin; and OC, osteocalcin. Although Col 1 mRNA levels are highest during the proliferation phase, matrix deposition continues to increase throughout the entire culture period (adapted from Owen *et al.*, 1990).

effects on the terminal differentiation of osteoblasts, as will be described later.

### The Osteoblast Lineage

Once positional signals have specified a given region of mesenchyme to become a skeletal structure, a series of events must be initiated leading either to initial formation of a cartilage anlage which is subsequently replaced by bone or, as is the case in the membranous bones, to direct recruitment of osteoblasts from neuroectoderm. In either instance, the conversion of a stem cell to a chondrocyte or osteoblast requires profound changes in gene expression. Clonal analysis of cells cultured from rat calvaria or marrow stroma demonstrated the presence of single cells having the potential to form the four mesenchymal phenotypes: muscle, fat, cartilage, and bone (Friedenstein *et al.*, 1987; Grigoriadis *et al.*, 1988). Similar multipotentiality is also observed when mesenchyme-derived cell lines such as C3H10T1/2 cells are treated with agents that affect DNA methylation (Taylor and Jones, 1979). These observations support a model in which all mesenchymal lineages are envisioned as being derived from a multipotential stem cell with unlimited capacity for self-renewal (Fig. 1). By a sequence of cur-

rently poorly defined events, stem cell progeny become committed progenitors to each of the four mesenchymal lineages. Osteoprogenitors are highly proliferative cells but are viewed as having a finite lifetime, making it essential that their numbers be renewed from multipotential stem cell populations (for reviews, see Aubin and Lui, 1996; Triffit, 1996).

As osteoprogenitors mature, they become increasingly restricted in terms of their proliferative potential. Because osteoprogenitors do not express proteins normally associated with osteoblasts (see below) and have no morphological characteristics to distinguish them from other mesenchymal cells, it is essential that unique antigenic markers be identified for the various early intermediates in this pathway. This type of approach has been crucial for identifying hematopoietic intermediates. Some recent progress has been made in identifying early markers for the osteoblast lineage. For example, the STRO-1 monoclonal antibody identified by Simmons and co-workers

defines a highly proliferative stromal cell population having the potential to form fibroblasts, adipocytes, and osteoblasts (Gronthos *et al.*, 1994). When STRO-1<sup>+</sup> cells are grown under conditions conducive to osteoblast formation (*i.e.*, in the presence of ascorbic acid, dexamethasone, and inorganic phosphate), mineralizing cultures develop that express osteoblast marker proteins. The identification of other early markers for this lineage will be important for further definition of the osteoprogenitor phenotype.

At present, BMPs are the only factors clearly shown to induce osteoblast differentiation in pluripotent mesenchymal cells. As noted above, BMP-2 is one of the proteins induced by *Shh* early in limb formation. In addition, several BMPs (BMP-2, 4, 6, and 7) stimulate osteoblast differentiation in cell populations likely to contain pluripotent mesenchymal cells, such as marrow stromal cells, mixed primary calvarial cultures, and mesenchymal cell lines (Thies *et al.*, 1992; Asahina *et al.*, 1993; Wang *et al.*, 1993; Gitelman *et al.*, 1995). In at least one case, the osteogenic activity of the BMP was irreversible (*i.e.*, cells retained their osteoblastic properties even after the BMP was removed), indicating that the pluripotent mesenchymal cells had become committed to the osteoblast lineage (Wang *et al.*, 1993). BMP-2 can also divert the differ-

entiation pathway of C2C12 myoblast from muscle to the osteoblast lineage (Katagiri *et al.*, 1994), although, in this study, effects of the BMP were reversible. Recent evidence suggests that glucocorticoids, a second class of compounds known to increase osteoprogenitors in marrow stromal cultures, may actually function by increasing BMP-6 synthesis (Boden *et al.*, 1997).

It is likely that BMPs are only one of several components necessary for the conversion of pluripotent stem cells to osteoprogenitors. For example, although BMP-2 clearly stimulates osteoblast differentiation in C3H10T1/2 mesenchymal cells, it also stimulates formation of adipocytes and chondrocytes, with adipocytes being preferentially induced at low BMP concentrations and chondrocytes and osteoblasts favored at high concentrations (Wang *et al.*, 1993). Interestingly, in this study, the activity of BMP-2 in stimulating formation of osteoblastic vs. adipogenic colonies could be perturbed by a number of humoral factors, including insulin, tumor-derived growth factor- $\beta$  (TGF- $\beta$ ), epidermal growth factor (EGF), leukocyte inhibitory factor (LIF), fibroblast growth factor-4 (FGF-4), and platelet-derived growth factor (PDGF). Other factors—such as the calcitropic hormone, 1,25-dihydroxyvitamin D<sub>3</sub>—stimulate induction of osteoblast characteristics, particularly in immature cells, and may, therefore, regulate progenitor formation (Franceschi *et al.*, 1988; Gerstenfeld *et al.*, 1996).

### **Expression of the Osteoblast Phenotype in Committed Osteoprogenitor Cells**

Osteoprogenitors, while restricted in their lineage potential, will differentiate into mature, secretory osteoblasts only if they undergo growth arrest and establish a collagenous extracellular matrix (ECM). In both primary osteoblast cultures and nontransformed osteoblast cell lines, expression of differentiation markers follows a clear temporal sequence (Fig. 2). Genes associated with cell proliferation—such as H4 histone, C-FOS, and C-MYC—are expressed at early times along with those encoding the pro $\alpha$ 1(I) and pro $\alpha$ 2(I) propeptides of type I collagen. Initial collagen matrix accumulation precedes and is essential for sequential expression of the differentiation-related proteins, alkaline phosphatase, the PTH/PTHrP receptor, bone sialoprotein, and osteocalcin (Gerstenfeld *et al.*, 1987; Owen *et al.*, 1990; Franceschi and Iyer, 1992; Ibaraki *et al.*, 1992; McCauley *et al.*, 1996). A similar pattern of gene expression is also seen *in vivo* and in organ culture; osteoprogenitors adjacent to bone are highly proliferative and express low levels of bone-specific proteins, while secretory osteoblasts on the bone surface stop dividing and produce large amounts of bone-specific ECM (Strauss *et al.*, 1990; Suva *et al.*, 1993). As will be discussed later in this article, a complete

model for transcriptional regulation in osteoblasts must explain the conversion of mesenchymal stem cells to osteoprogenitors as well as the influences of cell proliferation and ECM synthesis on gene expression.

### **General Models for the Control of Tissue-specific Gene Expression**

As noted above, the progression from uncommitted stem cell to osteoprogenitor and finally mature osteoblast and osteocyte is accompanied by specific changes in gene expression. A common theme in development is that specific transcription factors or groups of factors control the process of lineage commitment by selectively activating those genes to be expressed in the differentiated state. Several classes of tissue-specific transcription factors have been described, including the basic helix/loop/helix (bHLH) family involved in myogenic, myeloid, erythroid, and neuronal differentiation (Olson and Klein, 1994), bHLH leucine zipper factors involved in adipocyte and liver differentiation (Tontonoz *et al.*, 1993; Nishiyori *et al.*, 1994), orphan members of the steroid receptor superfamily such as HNF-4 involved in liver differentiation (Nishiyori *et al.*, 1994), and POU-domain factors involved in pituitary differentiation (Ingraham *et al.*, 1990).

Several experimental approaches have been successfully used to identify transcription factors controlling tissue-specific gene expression. These approaches can be divided into two general categories: (1) identification of factors able to confer a differentiated phenotype on an undifferentiated cell or tissue, and (2) identification of promoter elements able to confer tissue-specific expression on genes associated with a given phenotype, with subsequent identification and functional testing of nuclear proteins associating with these elements. The classic example of the first type of approach is the discovery of MyoD, a muscle-specific bHLH transcription factor. MyoD was isolated from a cDNA library enriched for transcripts present in a myogenic clone of C3H10T1/2 cells that were not present in wild-type undifferentiated cells. Transfection of MyoD into wild-type C3H10T1/2 cells induced myotube formation and muscle-specific gene expression (Weintraub *et al.*, 1991). MyoD was subsequently shown to bind to and activate skeletal muscle-related genes through a specific DNA element called an E-box and having the general sequence, CAnnTG. Four other myogenic bHLH factors (myogenin, myf-5, MRF-4, and myf-6) were subsequently cloned and shown to be involved in various steps in myoblast/myotube formation (Oldberg *et al.*, 1986). Although there is considerable redundancy between members of the MyoD family, gene knockout studies with one family member, myogenin, indicate that this factor is essential for the formation of

skeletal muscle (Olson and Klein, 1994). Similarly, neuroD, a second type of bHLH protein, can convert presumptive epidermal cells into neural cells when its mRNA is injected into *Xenopus* embryos (Lee *et al.*, 1995). An example of the second approach is the isolation of the hepatocyte-specific factor, HNF-1. This transcription factor was identified in hepatocyte nuclear extracts by its ability to bind to an oligonucleotide containing a sequence in the ornithine transcarbamylase gene promoter previously shown to be essential for liver-specific expression (Nishiyori *et al.*, 1994).

Although studies on osteoblast gene regulation have used both of the experimental approaches described above, investigators have achieved the greatest success in this area by focusing on specific gene promoters and associated nuclear factors. The osteocalcin gene and genes encoding the pro $\alpha$ 1(I) and pro $\alpha$ 2(I) chains of type I collagen have been the subject of intense investigation. Recently, several laboratories have also initiated studies on the bone sialoprotein gene. The following discussion will focus on the osteocalcin gene, which contains the best-understood osteoblast-specific promoter. Recent developments in our understanding of transcriptional control of the bone sialoprotein gene will also be briefly outlined. The type I collagen genes, which are subject to complex controls in a number of tissues in addition to bone, have been recently reviewed (Bornstein, 1996; Dodig *et al.*, 1996b) and will not be discussed in this article.

### **Transcriptional Regulation of the Osteocalcin Gene**

Of the major osteoblast-related gene products, osteocalcin exhibits the most selective pattern of expression; it is synthesized only by mature osteoblasts, odontoblasts, and cementoblasts (Bronckers *et al.*, 1987). Thus, control mechanisms governing its transcription are likely to include tissue-specific signals. Although the function of this  $\gamma$ -carboxyglutamic acid-containing protein is not completely understood, gene knockout experiments suggest a role in bone turnover (Ducy *et al.*, 1996). The first evidence that the osteocalcin gene promoter contained sufficient information to confer tissue-specific expression came from studies with transgenic animals. When 3.9 kb of untranscribed 5' sequence from the human osteocalcin promoter linked to the reporter gene, chloramphenicol acetyltransferase, was introduced into transgenic mice, selective expression was observed in osteoblasts (Kesterson *et al.*, 1993). This promoter region, therefore, contains sequences necessary for bone-specific expression. In recent years, investigators have studied the proximal promoter extensively, with the goal of identifying sequence elements necessary for basal and osteoblast-specific expression. These studies followed

two general paths: the identification and functional characterization of elements known to be binding sites for previously characterized transcription factors, and the search for new elements, particularly those able to confer an osteoblast-selective pattern of expression and bind nuclear factors preferentially expressed in osteoblasts.

Genes being actively transcribed in a particular tissue are known to exhibit enhanced sensitivity to nucleases such as DNase I. This increased sensitivity is thought to reflect absence of the usual nucleosomal chromatin structure and the presence of specific nonhistone proteins that are required for transcriptional activation. DNase I digestion of nuclei from ROS 17/2.8 osteoblast-like osteosarcoma cells identified two hypersensitive sites in the rat osteocalcin gene promoter, one between -590 and -390 nucleotides (nt) upstream from the transcription start site, and a second site between -170 and -70 nt (Montecino *et al.*, 1994). These sites were not found in nuclei from hepatoma cells which do not actively transcribe this gene. Inspection of these two regions revealed the presence of a number of sequence elements, including a functional vitamin D regulatory element (VDRE) and flanking activating protein-1 (AP-1) sites in the -590 to -390 nt region and a rich concentration of known transcription factor binding sites between -170 and -70 nt, some of which were shown to be active in functional assays. Within the downstream hypersensitive site are two E-box sequences, putative binding sites for the bHLH family of transcription factors (Tamura and Noda, 1994), two AP-1 sites, and a region called the osteocalcin box (-99 to -76) that contains a CCAAT motif and overlapping MSX-1 binding site (Lian *et al.*, 1989). Also included is a typical TATA box at -26 nt which serves as a binding site for the RNA polymerase-II-containing transcription initiation complex common to most eucaryotic promoters. Of these known transcription factor binding sites, particular attention has been paid to the VDRE and related AP-1 sites, E-boxes, and the MSX-1 site.

The osteocalcin gene was the first shown to be directly regulated by the steroid hormone, 1,25-dihydroxyvitamin D<sub>3</sub>, and was used to define the first functional VDRE (Kerner *et al.*, 1989; Markose *et al.*, 1990). The intracellular mechanism used by the 1,25-dihydroxyvitamin D<sub>3</sub> receptor complex to stimulate osteocalcin transcription and the interaction of this complex with other transcription factors has been recently reviewed (Blanco *et al.*, 1995; Stein *et al.*, 1996). Interestingly, recent studies have shown that this well-established response to the vitamin D hormone may be both developmentally regulated and unique to certain species. For example, a statistical analysis of the 1,25-dihydroxyvitamin D<sub>3</sub> response in cells from 12- vs. 17-day-old embryonic chick calvariae revealed that osteocalcin was induced by 1,25-

dihydroxyvitamin D<sub>3</sub> in younger, less mature cells and was inhibited by hormone in older, more osteoblast-like cells (Gerstenfeld *et al.*, 1996). Species differences have also been observed; acute 1,25-dihydroxyvitamin D<sub>3</sub> treatment induces osteocalcin in rat and human cells and reduces osteocalcin in mouse cells (Lian *et al.*, 1997). This difference is explained by sequence differences in the VDRE-containing region of mouse osteocalcin promoter that render it unresponsive to hormone and by indirect effects of 1,25-dihydroxyvitamin D<sub>3</sub> on the transcription factor, *Osf2* (see below) (Clemens *et al.*, 1997; R Zhang *et al.*, 1997).

The AP-1 sites bind members of the *fos/jun* family of transcription factors. Although they are not involved in the osteoblast-specific expression of the osteocalcin gene, AP-1 sites may be important for suppression of transcription during the proliferative phase of osteoblast development and complement the activity of osteoblast-specific factors to permit transcription after cell growth arrest (McCabe *et al.*, 1995; Stein *et al.*, 1996). Thus, *c-fos* and *c-jun* are expressed at high levels during the proliferative phase and decrease with cell growth arrest and differentiation, while *fra-1* and *junB* increase or remain at high levels during differentiation. Consistent with the hypothesis that *c-fos* may suppress osteocalcin transcription during the proliferative phase, overexpression of this protein by transfection of ROS17/2.8 cells with a *c-fos* expression vector inhibits osteocalcin expression (Schule *et al.*, 1990).

The presence of two E-box sequences within the proximal osteocalcin promoter suggested a possible role of bHLH transcription factors in osteoblast differentiation analogous to the *myoD* family of factors in muscle formation. Previous studies had, in fact, detected several non-tissue-specific bHLH proteins in osteoblasts and showed that they can be regulated by calcitropic factors (Ogata and Noda, 1991; Murray *et al.*, 1992, 1993; Ogata *et al.*, 1993; Benson *et al.*, 1995). In addition, forced expression of *Id 1*, an inhibitory bHLH protein known to interfere with the activity of tissue-specific bHLH proteins in other systems (Benezra *et al.*, 1990), was reported as partially blocking osteoblast differentiation and osteocalcin promoter activity (Murray *et al.*, 1992; Tamura and Noda, 1994). Mutagenesis of the downstream E-box (designated OCE1) at nucleotide -102 reduced osteocalcin promoter activity in ROS 17/2.8 cells by 50%. Furthermore, an uncharacterized putative bHLH protein present in nuclear extracts from osteosarcoma cells specifically bound this E-box. Binding activity which was low in undifferentiated mesenchymal cells was reported to be strongly induced by BMP-2 (Tamura and Noda, 1994). However, more recent studies showed that the nuclear factor binding to OCE1 is widely distributed in many non-osteogenic tissues and cell lines and has minimal

transcriptional activity when placed 5' to a minimal mouse osteocalcin promoter (Ducy and Karsenty, 1995). Furthermore, stable transfection of MC3T3-E1 mouse pre-osteoblast cells with the rat osteocalcin promoter containing a mutation in OCE1 did not affect the developmental upregulation of this promoter as cells differentiated in culture (Quarles *et al.*, 1997). Thus, bHLH proteins are not likely to play a major role in the osteoblast-specific expression of the osteocalcin gene, at least in cultured cells. However, this class of factors may be involved in earlier steps of bone formation. For example, during development, the recently discovered bHLH factor, *scleraxis*, is expressed at high levels within mesenchymal precursors of the axial and appendicular skeleton in a pattern that pre-figures cartilage anlage formation. *Scleraxis* levels then decline prior to overt cartilage formation and are not subsequently detected in osteoblasts (Cserjesi *et al.*, 1995). Thus, *scleraxis* or related factors may be involved in the early commitment of cartilage progenitors.

As noted above, homeobox genes play an important role in the specification of skeletal structures. The *MSX* genes (*MSX-1* and *MSX-2*) have demonstrated roles in craniofacial and tooth development. Furthermore, mutations in the *MSX-2* locus cause the autosomal-dominant disease, cranial synostosis (Jabs *et al.*, 1993; Satokata and Maas, 1994; Chen Y, *et al.*, 1996). For these reasons, the presence of an *MSX* site in the osteocalcin promoter was of particular interest. *MSX-2* mRNA is preferentially expressed in several osteoblast cell lines and primary cultures, while little or no message is detected in several types of non-osteoblastic mesenchymal cells (Hodgkinson *et al.*, 1993; Towler *et al.*, 1994; Heinrichs *et al.*, 1995). In addition, the *MSX* site in the rat osteocalcin promoter specifically binds recombinant *MSX-2* protein and related proteins present in ROS17/2.8 cell nuclear extracts. However, functional studies on the role of *MSX* proteins in osteocalcin gene expression are equivocal. On one hand, mutations in the *MSX* site of the rat osteocalcin promoter reduced transcriptional activity by approximately 50%, and suppression of *MSX-1* and -2 protein levels with antisense oligonucleotides partially inhibited mineralized nodule formation and osteocalcin levels in primary osteoblast cultures, yet forced expression of *MSX-2* suppressed promoter activity (Towler *et al.*, 1994). Interpretation of these results is further complicated by the fact that *MSX* proteins are known to participate in protein:protein interactions with other homeoproteins such as the *Dlx* family and can inhibit transcription by binding to other transcription factors rather than acting through a specific DNA element (H Zhang *et al.*, 1997). In conclusion, although *MSX* proteins are clearly expressed in osteoblasts and contribute to the activity of the osteocalcin promoter, other factors are clearly

required to explain the highly bone-selective pattern of expression of this gene. These results are in contrast to recent transgenic analysis of the pro $\alpha$ 1(I) collagen promoter, which identified a 13-bp region between -1683 and -1670 bp containing an Msx2 binding site that was essential for expression in osteoblasts (Dodig *et al.*, 1996a).

To gain a more complete understanding of gene expression in bone, Ducy and Karsenty initiated a series of investigations to examine, systematically, the proximal 1.3 kb of the mouse osteocalcin promoter for osteoblast-specific elements (Ducy and Karsenty, 1995). Murine and rodent osteocalcins are encoded in two genes. In mice, osteocalcin genes 1 and 2 are contained in a gene cluster which also contains a third member, osteocalcin-related gene, which is expressed only in the kidney (Desbois *et al.*, 1994). Systematic deletion analysis of the osteocalcin gene 2 promoter in ROS 17/2.8 cells revealed no major loss of activity between -1300 and -147 bp from the transcription start site (Ducy and Karsenty, 1995). The -147 promoter retained information necessary for osteoblast-specific expression, since it was expressed at high levels in ROS17/2.8 cells, but showed low to undetectable activity in C2/C12 myoblasts, F9 teratocarcinoma cells, and S194 myeloma. Further deletion to -34 bp completely abolished promoter activity. DNase I protection assays with ROS 17/2.8 nuclear extracts and the -147 promoter revealed three major protected regions, designated osteoblast-specific element 1 (OSE1) between -64 and -47 nt, OSE2 between -146 and -132 nt, and OCE1 (which contains the E-box described above) between -110 and -83 nt. Gel retardation assays with double-stranded oligonucleotides containing each sequence and nuclear extracts from ROS cells and primary mouse osteoblasts revealed that both OSE1 and OSE2 specifically bound nuclear factors, designated osteoblast-specific factor 1 (Osf1) and Osf2, that were selectively expressed in osteoblast-like cells. In contrast, OCE1 bound a factor or factors expressed in a wide variety of cell types. Deletions in the -147 bp promoter that removed OSE2 and OSE1 drastically reduced promoter activity, while OCE1 deletion was without effect. Furthermore, multiple copies of either OSE1 or OSE2 selectively stimulated activity of the minimal -34 bp promoter in osteosarcoma cells (greater than 200-fold stimulation), but not in C2/C12 or F9 cells, while minimal activity was detected with multimers of the OCE1 sequence. Mutational analysis defined the minimal OSE2 sequence as CCAACCAC. This study provided the first strong functional evidence that osteoblast-specific enhancer sequences were present in the osteocalcin promoter.

The initial clue to the identification of the transcription factor binding OSE2 came from the laboratories of Stein and Lian, who had previously identified two sequences between -605 and -599 nt and -459 and -434 nt in the rat osteocalcin promoter that specifically bound

osteoblast nuclear proteins. The factor binding these two regions was called nuclear matrix protein 2 (NMP2), because it could be extracted from this nuclear fraction. The NMP2 component interacting with the -605 to -599 region was detected in osteoblast-like cells, but not hepatoma, promyelocytic leukemia, or cervical carcinoma cells (Bidwell *et al.*, 1993). Methylation protection assays identified the upstream NMP2 binding site as 5'-ACCACT-3'. This sequence was originally thought to be a binding site for CCAAT box-binding factors, but, as was later pointed out, it also resembles the consensus sequence for the AML/*runt* domain family of transcription factors which bind the DNA consensus sequence: 5'-PuCCPuCA-3' (Merriman *et al.*, 1995). Pebp2 (polyoma enhancer binding protein) is the murine homologue of the human acute myelogenous leukemia (AML) transcription factor family. The rat osteocalcin promoter also contains a third downstream AML/*runt* site equivalent to the OSE2 site previously defined in the mouse promoter (Ducy and Karsenty, 1995). Although the two upstream NMP2 binding sites of the rat osteocalcin promoter served as the basis for the first identification of AML/*runt* domain transcription factors in bone, to date neither site has been shown to have functional activity in stimulating osteocalcin promoter activity. In the mouse promoter, the most 5' site between -608 and -602 nt is not necessary for osteoblast-specific activity *in vivo* (Frendo *et al.*, 1998). Subsequent analysis by both the Karsenty (Baylor College of Medicine, Houston) and Stein/Lian laboratories (University of Massachusetts Medical School, Worcester) showed that the downstream AML site could bind *in vitro*-translated AML1 protein (Geoffroy *et al.*, 1995; Banerjee *et al.*, 1996). Furthermore, AML1 activated transcription of osteocalcin promoter-reporter gene constructs when transfected into fibroblasts or F9 teratocarcinoma cells. Finally, the AML-like protein in osteoblast nuclear extracts could interact with an anti-AML1 antibody, indicating that the bone protein was a member of this family. AML-like proteins had previously been extensively characterized in both humans and mice, principally because a chromosomal translocation in the AML1 locus is frequently found in human leukemias (Meyers *et al.*, 1993). These transcription factors are related to the *Drosophila* pair-rule protein, *runt*, a molecule required for insect segmentation (Gergen and Wieschaus, 1985). All function as dimers of  $\alpha$  and  $\beta$  subunits. Three  $\alpha$  subunits have been identified and given separate designations in humans and mice: Human AML1 corresponds to murine Pebp2 $\alpha$ B/Cbfa2, AML2 to murine Pebp2 $\alpha$ C/Cbfa3, and AML3 to Pebp2 $\alpha$ A/Cbfa1. In addition, two  $\beta$  subunits, Pebp2 $\beta$ 1 and Pebp2 $\beta$ 2, have been identified in the mouse and form dimers with the  $\alpha$  subunits that are obligatory for transcriptional activation (Sasaki *et al.*, 1996).

Definitive identification of the AML-like protein in osteoblasts was recently provided by the Karsenty labora-

tory (Ducy *et al.*, 1997). Using low-stringency hybridization of an osteoblast cDNA library with a Cbfa1 *runt* domain probe, they isolated a cDNA, sequenced it, and showed it to be identical in its 3' end to Cbfa1 (AML3), a molecule originally cloned from a T-cell library. The protein-coding portion of this sequence contains both the proline/serine/threonine-rich and *runt* domains of Cbfa1. More detailed analysis identified a 19-amino-acid N-terminal region in the *Osf2/Cbfa1* sequence cloned from osteoblasts that was not reported in the original Cbfa1 cDNA (Thirunavukkarasu *et al.*, 1998). Extensive structure/function analysis of *Osf2/Cbfa1* identified several domains necessary for transcriptional activity, including a *myc*-related nuclear localization signal, three transcriptional activation domains, and a repression region. *Osf2/Cbfa1* has several properties expected of an osteoblast-specific transcriptional regulator, including ability to activate the osteocalcin and osteopontin promoters through an AML sequence, selective expression in osteoblasts and osteosarcoma cells, upregulation in mesenchymal cells after induction of osteoblast differentiation with BMPs, and, most importantly, ability to induce multiple osteoblast marker genes [ $\text{pro}\alpha 1(\text{I})$  collagen, osteopontin, bone sialoprotein, and osteocalcin] when transfected into mesenchymal cell lines such as C3H10T1/2 cells or undifferentiated murine MC3T3E1 pre-osteoblast cells. In addition, blocking *Osf2/Cbfa1* expression with antisense oligonucleotides inhibited  $\text{pro}\alpha 1(\text{I})$  collagen, osteopontin, and osteocalcin expression and mineralized nodule formation (Banerjee *et al.*, 1997; Ducy *et al.*, 1997). Of further interest, binding sites for Cbfa1 were detected in promoter regions of osteoblast marker genes, although it has not yet been demonstrated that all of these sites are functional. In fact, *Osf2/Cbfa1* may actually regulate some osteoblast-related genes through an indirect mechanism (see below).

A final intriguing property of AML family members is their ability to interact with the nuclear matrix, a filamentous ribonucleoprotein network thought to provide the underlying structure for higher-order nuclear organization into RNA polymerase II-containing transcription foci (Nickerson *et al.*, 1995). A recent study by Zeng and co-workers identified a 31-amino-acid region in AML1B necessary for nuclear matrix binding that could be distinguished from the DNA binding domain (Zeng *et al.*, 1997). The presence of a related sequence in AML3 (*Osf2/Cbfa1*) suggests that this transcription factor may activate genes like osteocalcin by controlling their chromatin organization with respect to the transcriptional apparatus.

### **Role of *Osf2/Cbfa1* in Osteoblast Development and Genetic Diseases of Bone**

While it is not clear that *Osf2/Cbfa1* alone can induce the

full repertoire of osteoblast activities in mesenchymal cells (*i.e.*, direct induction of all known markers and formation of a mineralized ECM), this molecule clearly is a major determinant of the osteoblast phenotype. *In situ* hybridization studies of developing mouse embryos first detected *Osf2/Cbfa1* mRNA in mesenchymal condensations of the axial and appendicular skeleton and skull on embryonic day 12.5 (Ducy *et al.*, 1997). A similar pattern of  $\beta$ -galactosidase expression was found in transgenic animals containing a LacZ insert in the CBFA1 locus (Otto *et al.*, 1997). The *Osf2/Cbfa1*-positive cells at this stage also contain markers for initial cartilage condensations which suggest that they represent a common precursor for both chondrocytes and osteoblasts. At later times (embryonic days 14.5 and 16), *Osf2/Cbfa1* is found only in osteoblast precursors.

In the same exciting issue of the journal, *Cell*, that reported the cloning of *Osf2/Cbfa1*, two papers described the phenotype of CBFA1-deficient mice (Komori *et al.*, 1997; Otto *et al.*, 1997), and a third demonstrated that mutations in the human gene cause the genetic disorder, cleidocranial dysplasia (Otto *et al.*, 1997). CBFA1-null mice were generated by homologous recombination, and both heterozygous and homozygous animals were found to have severe skeletal abnormalities. The phenotype of the CBFA1<sup>-/-</sup> animals was particularly remarkable: Animals were smaller than their heterozygous or wild-type littermates, and their skeletons were almost completely devoid of mineralized bone. In contrast, cartilage formation was not grossly affected by the mutation. The skeletons of heterozygotes exhibited a severe hypoplasia of the clavicle and delayed development of the membranous bones of the skull, resulting in open anterior and posterior fontanelles and wide cranial sutures. *In situ* hybridization and Northern blot analysis failed to detect osteocalcin in the osteoblasts of CBFA1<sup>-/-</sup> animals. Interestingly, osteocalcin and alkaline phosphatase could be induced in calvarial cells from mutant animals if they were treated with high concentrations of BMP-2 (Komori *et al.*, 1997). This suggests that factors in addition to Cbfa1 play important roles in the induction of these genes by BMPs, at least in cell culture.

The phenotype of heterozygotic CBFA1 mutants is remarkably similar to the human disease, cleidocranial dysplasia (CCD), an autosomal-dominant disorder characterized by the absence or near-absence of the clavicles, open fontanelles, supernumerary teeth, and short stature. In fact, two lines of evidence now show that the CBFA1 gene is mutated in this disease. First, the CBFA1 locus on mouse chromosome 17 is deleted in the *Ccd* mouse, a radiation-induced mutant with a phenotype similar to human CCD patients (Otto *et al.*, 1997). Second, the human CCD locus and CBFA1 have been co-localized to chromosome 6p21, and several CCD families have



been identified in which the CBFA1 gene is deleted or contains insertion, deletion, or missense mutations (Lee *et al.*, 1997; Otto *et al.*, 1997). The conclusion from these studies is that CCD exhibits a dominant form of inheritance, because it is caused by loss of function mutations. These are generally lethal in homozygotes and cause CCD in heterozygotes because of haplo-insufficiency.

### **Regulation of the Bone Sialoprotein Gene**

Bone sialoprotein, a highly acidic bone phosphoprotein with a possible role in ECM mineralization (Hunter and Goldberg, 1993), is also preferentially expressed in osteoblasts, although it has a somewhat broader tissue distribution than osteocalcin. BSP is found at high levels in mature osteoblasts, but not their immature precursors, hypertrophic chondrocytes, odontoblasts, cementoblasts, and, to a lesser extent, osteoclasts, trophoblasts, and certain metastatic cancers (Bianco *et al.*, 1991; Chen *et al.*, 1993; MacNeil and Somerman, 1993; Bellahcene *et al.*, 1996). The BSP gene has been cloned from rat (Li and Sodek, 1993), mouse (Benson *et al.*, 1997), human (Kerr *et al.*, 1993; Kim *et al.*, 1994), and avian species (Yang and Gerstenfeld, 1997). An approximately 2.7-kb sequence of the rat BSP promoter driving expression of a firefly luciferase reporter gene was analyzed in transgenic animals and was shown to be selectively expressed in bone osteoblasts, with negligible expression in the kidney, liver, stomach, intestine, and spleen (Chen J, *et al.*, 1996). Thus, important elements for osteoblast-selective expression are contained in this promoter region. Inspection of the proximal BSP promoter from mammalian species reveals a high degree of sequence conservation, particularly in the first approximately 370 nt upstream from the transcription start site (*ca.* 75% sequence identity across rat, mouse, and human promoters). This region contains a number of previously characterized DNA sequences, including an inverted TATA box with overlapping VDRE, a CCAAT box, a type II collagen-like silencer, and putative binding sites for the transcription factors CREBP (cAMP response element binding protein), NF $\kappa$ B, and fos/jun (AP-1), as well as two homeobox domains (MSX-2 and Engrailed-1) (Sodek *et al.*, 1996; Yang and Gerstenfeld, 1997). The VDRE and a distal glucocorticoid regulatory element have both been shown to have modest activity *in vivo* and in cell culture (Ogata *et al.*, 1995; Sodek *et al.*, 1995). The chicken BSP promoter is also stimulated approximately five-fold by PTH, and this response is mediated by a CRE half-site (Yang and Gerstenfeld, 1996). Although the chicken promoter shares less overall homology with mammalian BSP promoters, many of the above-listed regulatory elements are conserved, including the two homeobox sites and the collagen silencer region, suggesting that they have important roles in regulation (Yang and Gerstenfeld, 1997).

Only recently has substantial progress been made in the functional definition of specific elements in the BSP promoter necessary for osteoblast-specific expression. Initial studies in which 2.7 kb of the rat promoter was transiently transfected into osteoblast-like vs. fibroblastic cells revealed little preferential expression in osteoblasts (Sodek *et al.*, 1994). More recent studies with a 1.2-kb fragment of the chicken promoter showed preferential expression in osteoblasts and cephalic chondrocytes when compared with skin fibroblasts (Yang and Gerstenfeld, 1997). Some of this specificity was attributed to a suppression of promoter activity in fibroblasts, possibly *via* the collagen II silencer in the region between -966 and -949 bp. Preliminary studies from the author's laboratory show approximately 20-fold higher expression of a 2.5-kb fragment of the mouse BSP promoter in osteoblasts relative to C2/C12 myoblasts, 3T3-L1 adipocytes, F9 teratocarcinoma cells, and S194 leukemia cells (Benson *et al.*, 1997). Deletion analysis revealed much of the tissue specificity to reside in a region between -173 and -334 nt upstream from the transcription start site. Deletion of the region containing the collagen II silencer did not increase promoter activity in non-bone cells. Of further interest, although consensus binding sites for AML family members are present in the BSP promoter from all species examined, none is contained in the -173 to -334 region. This suggests that the induction of BSP mRNA by *Osf2/Cbfa1*, noted above (Ducy *et al.*, 1997), may be through an indirect mechanism.

### **Extracellular Matrix Regulation of Osteoblast-specific Gene Expression**

The extracellular matrix (ECM) plays an essential if often overlooked role in osteoblast differentiation. As a cell primarily devoted to matrix production, the osteoblast must have the ability to monitor the composition of the ECM it is secreting as well modify matrix production to accommodate the changing mechanical needs of bone. It is not known how this is accomplished. However, there is now strong evidence that osteoblast differentiation and collagen matrix secretion are closely linked. An appreciation for the significance of the ECM in osteoblast function may be gained from an examination of the requirement for ascorbic acid (AA, reduced vitamin C) in osteoblast differentiation. AA is an essential co-factor for hydroxylation of procollagen chains, a necessary step for triple helix formation, secretion, and collagen fibril assembly. Thus, in vitamin C deficiency, collagen matrix production is essentially blocked. *In vivo*, both bone formation and osteoblast differentiation, as assessed by expression of osteocalcin and alkaline phosphatase mRNAs, are severely reduced in animals on vitamin-C-deficient diets (Togari *et al.*, 1995; Mahmoodian *et al.*,

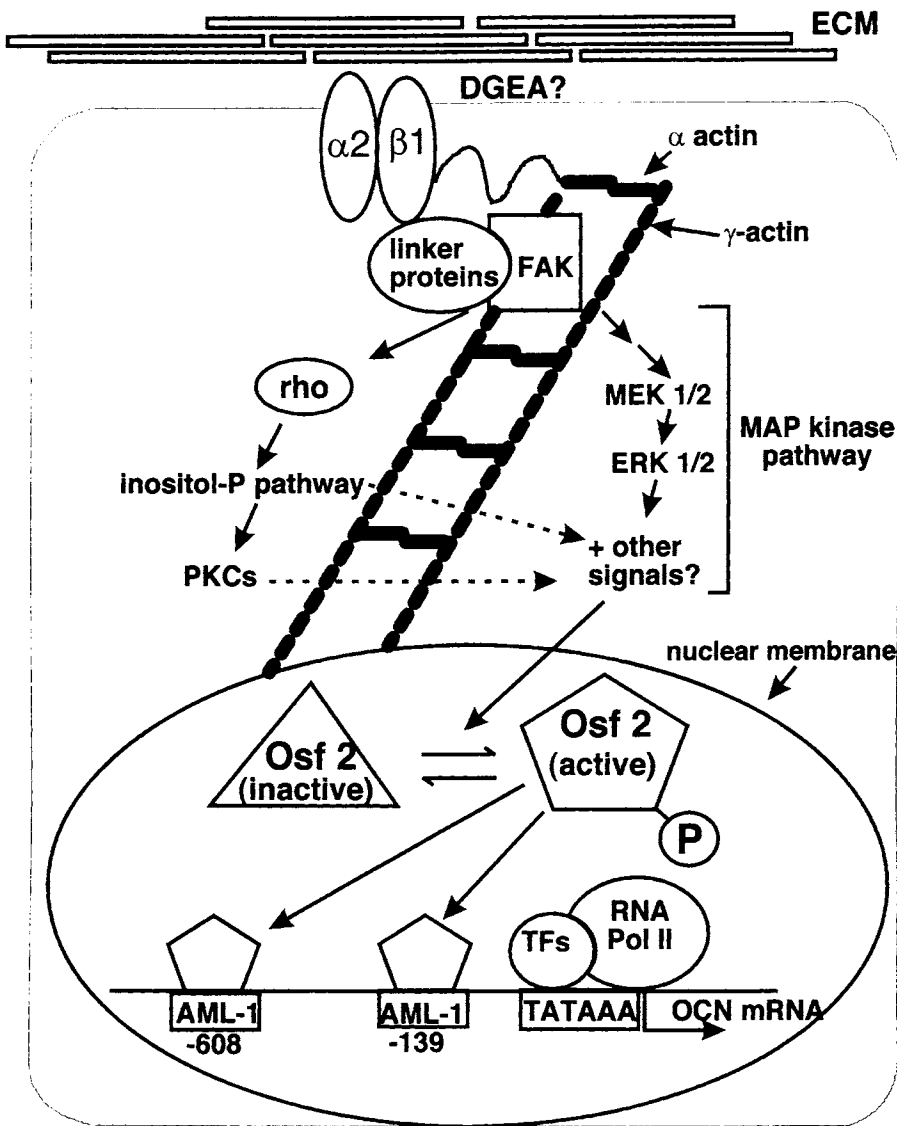
1996). This requirement is also seen *in vitro*, where AA is essential for differentiation and mineralization of primary osteoblast cultures and nontransformed osteoblast-like cell lines such as murine MC3T3-E1 cells (Gerstenfeld *et al.*, 1987; Owen *et al.*, 1990; Franceschi and Iyer, 1992; Ibaraki *et al.*, 1992; Quarles *et al.*, 1992; McCauley *et al.*, 1996). AA responsiveness is a unique property of non-transformed cells and is not observed in osteosarcoma cell lines such as the ROS17/2.8 cells used in many of the studies described above to characterize the osteocalcin promoter. These cells express high levels of osteoblast markers in the absence of AA, which suggests either that an important level of regulation has been lost in this osteosarcoma cell line or that ROS17/2.1 cells have progressed beyond the point in the osteoblast lineage where ECM synthesis is required for phenotypic expression. Studies from the author's laboratory have demonstrated that actions of AA on osteoblast differentiation are mediated by the collagenous matrix (Franceschi and Iyer, 1992; Franceschi *et al.*, 1994). Disruption of matrix secretion with specific inhibitors or enzymatic digestion of secreted collagen blocks the induction of osteoblast-related genes. A similar requirement for collagen synthesis was recently demonstrated in retinoic-acid-treated ROB-C26 cells, a multipotential mesenchymal cell line (Shi *et al.*, 1996).

Studies on the genetic disease osteogenesis imperfecta (OI) further support the importance of the ECM environment in osteoblast function. The majority of OI mutations are in glycines that occupy every third residue in the helical region of type I collagen or are deletions in the triple helical domain. The consequences of these mutations may, to various degrees, be similar to the situation encountered in AA deficiency: Procollagen triple-helix structure is destabilized with accompanying decreased rates of secretion, processing and cross-linking, and abnormal fibril assembly. These defects would, in turn, compromise the ability of cells to differentiate normally. In fact, osteopenia due to reduced extracellular matrix synthesis is a hallmark of OI, and osteoblasts cultured from OI patients exhibit reduced synthesis of collagen and proteoglycan (Fedarko *et al.*, 1992, 1996). To examine more directly the effect of abnormal collagen structure on osteoblast differentiation, Wenstrup and co-workers created MC3T3-E1 cell lines that stably expressed a human pro $\alpha$ 1(I) collagen minigene with a truncated triple helical domain known to cause an OI phenotype in transgenic mice (Wenstrup *et al.*, 1996). As predicted from studies with AA-deficient cells, osteoblasts containing this abnormal collagen exhibited rapid matrix turnover, severely abnormal collagen fibrillogenesis, and decreased levels of alkaline phosphatase and bone sialoprotein. However, osteocalcin levels were actually elevated, indicating that this matrix abnormality affects osteocalcin differently from other osteoblast markers.

More recent studies focused on the relationship between matrix synthesis and induction of osteoblast-specific gene expression, with the well-characterized mouse osteocalcin gene 2 as a model (Xiao *et al.*, 1997). ECM synthesis increased promoter activity approximately 20-fold in MC3T3-E1 cells. Significantly, this response was shown to require OSE2, the downstream promoter binding site for the transcription factor Osf2/Cbfa1. Mutation or deletion of this element abolished activity. Furthermore, OSE2 oligomers in the absence of other promoter elements were able to confer matrix responsiveness on a -34-nt minimal osteocalcin promoter construct. Although deletion analysis suggested that other upstream regions of the osteocalcin promoter between -657 and -147 may also contribute to ECM responsiveness, the downstream OSE2 site is clearly essential, because a point mutation in this element abolished the AA responsiveness of a -657 promoter construct. AA treatment was also accompanied by a five-fold increase in the binding of Osf2/Cbfa1 to OSE2. Thus, ECM synthesis stimulates osteoblast-specific transcriptional activity in part by activating/upregulating the tissue-specific transcription factor, Osf2/Cbfa1. We do not currently know if this mechanism explains the upregulation of other genes by AA. However, like osteocalcin, the bone sialoprotein promoter is upregulated by AA and may be subjected to similar controls (Benson *et al.*, 1997).

Ongoing studies in the author's laboratory and elsewhere are exploring possible mechanisms to explain how the collagenous ECM can stimulate osteoblast-specific gene expression. Several years ago, we proposed a general model for ECM regulation of osteoblast differentiation in which the matrix was viewed as interacting with the cell surface through specific integrins to initiate intracellular signaling pathways leading to osteoblast-specific gene expression (Franceschi, 1992). Integrins are the principal mediators of the molecular dialogue between a cell and its ECM environment and are crucial to processes such as tissue morphogenesis, wound healing, and cell migration and attachment (for reviews, see Clark and Brugge, 1995; Gumbiner, 1996). All function as dimers composed of an  $\alpha$  and a  $\beta$  subunit. The unique combinations of subunits determine which ECM molecules will be recognized by a cell. For example, type I collagen interacts with both  $\alpha$ <sub>1</sub> $\beta$ <sub>1</sub> and  $\alpha$ <sub>2</sub> $\beta$ <sub>1</sub> integrins, while fibronectin binds  $\alpha$ <sub>3</sub>,  $\alpha$ <sub>4</sub>, and  $\alpha$ <sub>5</sub>: $\beta$ <sub>1</sub> dimers. Integrins convey information about the extracellular environment both by serving as a direct physical link between the ECM and actin cytoskeleton *via* the proteins, talin, vincullin, and paxillin, and by functioning as signal transduction molecules to stimulate tyrosine phosphorylation cascades involving the mitogen-activated protein kinase (MAP kinase) and other pathways (Roeckel and Krieg, 1994; Sudbeck *et al.*, 1994; Lin *et al.*, 1997).

Recently, considerable evidence has accumulated



supporting a role for integrins in osteoblast differentiation. Osteoblasts are now known to express several integrin species, including  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_4$ ,  $\alpha_5$ ,  $\alpha_v$ ,  $\beta_1$ ,  $\beta_2$ , and  $\beta_3$  subunits (Clover *et al.*, 1992; Hughes *et al.*, 1993; Grzesik and Gehron-Robey, 1994; Saito *et al.*, 1994; Sinha and Tuan, 1996). Furthermore, disruption of integrin:ECM interactions, specifically those involving collagen and fibronectin, blocks osteoblast differentiation and mineralization. Thus, addition of antibodies to  $\alpha_2$  integrin or a DGEA peptide which is a component of the cell-binding domain of type I collagen (Staatz *et al.*, 1991) blocks AA-dependent induction of alkaline phosphatase and differentiation-dependent down-regulation of TGF- $\beta$  receptors in MC3T3-E1 cells (Takeuchi *et al.*, 1996). These responses appear to require focal adhesion kinase and the MAP kinase pathway, because selective inhibition of either of these intermediates blocks alkaline phosphatase induction (Takeuchi *et al.*, 1997). In separate studies, a peptide containing the RGD cell-binding domain of fibronectin was shown to inhibit both bone formation and resorption in a mineralizing organ culture system (Gronowicz and Derome, 1994). Similarly, long-term culture of calvarial osteoblasts with anti-fibronectin antibodies or fibronectin peptides containing the RGD cell-binding domain has also been reported to suppress differentiation (Moursi *et al.*, 1996). The author's laboratory recently showed that disruption of  $\alpha_2$  integrin function by both anti- $\alpha_2$  integrin antibody and DGEA peptide also blocks ascorbic-acid-dependent induction of the osteocalcin promoter, osteoblast differentiation, and activation of Osf2/Cbfa1 (Xiao *et al.*, 1998a). The same concentrations of anti- $\alpha_5$  integrin antibody or RGD-containing peptide were inactive, although the RGD peptide was inhibitory at higher concentrations. Interestingly, the dramatic matrix-dependent increase in Osf2/Cbfa1 binding to OSE2 noted above is not accompanied by parallel changes in the level of transcription factor mRNA or protein. This suggests that the ECM induces a modification in Osf2/Cbfa1 to increase its activity as a transcriptional enhancer rather than

**Figure 3.** Model for extracellular matrix activation of the osteocalcin promoter. Key components of this model are: (1) the interaction of the type I collagen matrix with the  $\alpha_2\beta_1$  integrin, followed by signaling to the nucleus; (2) phosphorylation and activation of Osf2/Cbfa1 and possibly other transcription factors; and (3) binding of Osf2/Cbfa1 to AML sites in the osteocalcin promoter and stimulation of transcription. Several known integrin-mediated signal transduction pathways are shown (Clark and Brugge, 1995; Gumbiner, 1996), although those relevant to osteoblast function have not been defined. Also indicated is the physical linkage between integrins and the cytoskeleton via  $\alpha$ -actinin and the actin filaments, which may also control transcription. Not shown is the possible role of nuclear organization in the activation of Osf2/Cbfa1. It has been proposed that this factor can associate with the nuclear matrix as well as with AML sites in the osteocalcin promoter, thereby bringing the promoter into contact with the basal transcription initiation complex. Abbreviations:  $\alpha_2\beta_1$ , integrin subunits known to form a receptor for type I collagen; DGEA, Asp-Gly-Glu-Ala sequence of type I collagen, reported to interact with the  $\alpha_2\beta_1$  integrin; FAK, focal adhesion kinase; Osf2, Osf2/Cbfa1 transcription factor; AML, DNA binding site for AML/*runx* transcription factors (positions in the mouse osteocalcin gene 2 promoter are indicated); and TFs, components that, together with RNA polymerase II, form the basal transcription initiation complex.

increasing protein levels. In a related study, overexpression of the  $\alpha_2$  integrin subunit in primary calvarial osteoblast cells also greatly increased osteocalcin promoter activity (Zimmerman *et al.*, 1997).

Recent preliminary results from the author's laboratory indicate that *Osf2/Cbfa1* is phosphorylated by MAP kinases and that this modification stimulates transcriptional activity (Xiao *et al.*, 1998b).

Taken together, these results strongly support a model in which an  $\alpha_2\beta_1$  integrin: type I collagen interaction is necessary for phosphorylation and activation of osteoblast-specific transcription factors like *Osf2/Cbfa1* present in committed osteoprogenitor cells. There are several examples of type I collagen:  $\alpha_2\beta_1$  integrin interactions being important in the regulation of other cellular activities, such as activation of collagenase (Riikonen *et al.*, 1995), stimulation of collagen gel contraction (Schiro *et al.*, 1991), and kidney tubule formation (Schwimmer and Ojakian, 1995), although in no case has the entire integrin-mediated pathway been delineated. Clearly, a major direction for future research will be to identify the signal transduction pathway linking integrin:ECM binding to changes in osteoblast-specific gene expression and to determine how this pathway is regulated by changes in matrix synthesis and turnover, mechanical loading of bone, and the hormonal milieu. Fig. 3 summarizes our current thinking about how the ECM regulates gene expression in osteoblasts.

### **Is the Tooth a Kind of Bone?**

At the molecular level, dentin and cementum share many similarities with bone, including the presence of a mineralized type I collagen matrix and noncollagenous proteins such as osteocalcin, bone sialoprotein, alkaline phosphatase, and BMPs (MacNeil and Somerman, 1993; Lesot *et al.*, 1994). In fact, because no unique markers have been identified to distinguish it from bone, it has been proposed that the cementoblast may actually be a specialized type of osteoblast (MacNeil *et al.*, 1998). In contrast, odontoblasts do express at least two unique proteins, dentin sialoprotein and dentin phosphoprotein, which were recently shown to be synthesized as a single transcript (MacDougall *et al.*, 1997). Like osteoblasts, cementoblasts and odontoblasts require ascorbic acid to express a differentiated phenotype (D'Errico *et al.*, 1997; Heimrika-Wagner *et al.*, 1982), and it is likely that cell:matrix interactions shown to be crucial for osteoblast differentiation are also necessary for dentin and cementum formation. A central question concerns the degree to which genetic mechanisms controlling osteoblast differentiation are relevant to our understanding of odontoblasts and cementoblasts. For example, is *Osf2/Cbfa1* an important factor for determination of these cell types, or are other transcription factors involved? Individuals with cleidocranial dysplasia have

teeth—extra teeth in fact—but this may be a consequence of their heterozygous status for the *CBFA1* gene. *CBFA1*<sup>-/-</sup> mice contain hypoplastic tooth primordia, although the appropriate cell layers of differentiating tooth structures were present (Otto *et al.*, 1997). It was not determined whether these structures express osteocalcin or other matrix proteins. A comparison of the transcriptional regulation of genes like osteocalcin, bone sialoprotein, and dentin sialophosphoprotein in bones with that in teeth may reveal important information about more subtle regulatory mechanisms that distinguish these two similar, yet functionally very different, tissues.

### **Conclusions and Future Directions**

The organogenesis of skeletal structures can be divided into two broad phases: the establishment of a morphogenic field in which signals from organizing centers like the ZPA specify in time and space which cells are destined to form bone, followed by a differentiation phase when gene expression patterns are altered in response to coded positional information to produce chondrocytes and osteoblasts at sites of overt bone formation. Although this article focused on gene regulation during osteoblast differentiation, much progress has also been made in our understanding of these early morphogenic signals. The combined use of promoter analysis and transgenic technology has allowed our understanding of gene expression in bone to progress rapidly. A striking example of this rapid pace of discovery was the simultaneous publication of the identification, targeted inactivation, and human genetics of *Osf2/Cbfa1*. Undoubtedly, this rate of progress will only increase in the coming years. Some of the more outstanding research questions to be addressed in the next five years are summarized below:

#### **(i) HOW DO EARLY PATTERNING EVENTS INITIATED BY FACTORS LIKE SHH LEAD TO THE FORMATION OF BONE AT SPECIFIC SITES?**

Hox gene expression is known to precede mesenchymal condensations in regions targeted for future bone formation, but it remains to be determined how these sites are specified and distinguished from areas destined to form non-osseous structures.

#### **(ii) IS OSF2/CBFA1 THE ONLY TRANSCRIPTION FACTOR REQUIRED FOR OSTEOBLAST-SPECIFIC GENE EXPRESSION, OR IS IT ONLY ONE OF SEVERAL COMPONENTS, PERHAPS AN ENTIRE FAMILY OF OSTEOGENIC NUCLEAR FACTORS, THAT WORK IN A CONCERTED FASHION TO CONTROL TRANSCRIPTION?**

An understanding of the full range of transcriptional controls necessary for osteoblast-specific gene expres-

sion will require the systematic analysis of multiple gene promoters.

### **(III) WHAT ARE THE RELEVANT PATHWAYS FOR ACTIVATION OF OSF2/CBFA1?**

Osteoblast cultures grown in the absence of AA contain Osf2/Cbfa1, but the transcriptional activity of this factor remains low until cells are allowed to synthesize an ECM. Preliminary studies suggest the involvement of an integrin-mediated event for Osf2/Cbfa1 activation, but the details of this pathway remain to be determined.

### **(IV) ARE THE TRANSCRIPTIONAL CONTROLS IDENTIFIED IN BONE FOUND IN OTHER MINERALIZED TISSUES SUCH AS TEETH?**

Initial reports on tooth formation in Osf2/Cbfa1 knockout mice suggest the involvement of this factor in tooth morphogenesis, but it remains to be determined if it functions similarly in bones and teeth. Elucidation of the regulatory controls for multiple odontoblast-related genes will be required to discriminate between general and tooth-specific gene regulatory mechanisms.

### **Acknowledgments**

This work was supported by National Institute of Dental Research grants DE-11723 and DE-12211. The author thanks Mr. M. Douglas Benson for the preparation of Figs.

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