Functional Cooperativity Between Osteoblast Transcription Factors: Evidence for the Importance of Subnuclear Macromolecular Complexes?

R. T. Franceschi

Department of Periodontics/Prevention/Geriatrics, School of Dentistry, Department of Biological Chemistry, School of Medicine,
The University of Michigan, Ann Arbor, MI 48109-1078, USA

Abstract. The accompanying article by Drs. Lian and Stein describes current thinking on how genes are organized in the nucleus and suggests that subnuclear localization is critical for the control of gene expression in bone. In particular, it is proposed that a major function of the osteoblast transcription factor, Runx2, is to tether genes that are active in osteoblasts to the nuclear matrix and serve as an organizing center for other nuclear factors which together form osteoblast-specific transcriptional units. Although it is still not established that the nuclear matrix localization function of Runx2 is essential for all its biological activities, there is no question that this factor plays a central role in mediating the response of osteoblasts to a variety of signals, as would be expected if Runx2 were involved in organizing the transcriptional apparatus. As will be discussed, Runx2 is required for the response of osteoblasts to other lineage-specific transcription factors and signals initiated by extracellular matrix-integrin binding, growth factors, hormones, and morphogens. We hypothesize that Runx2 transduces this wide range of responses by cycling between active, phosphorylated and a less active, dephosphorylated states which can selectively interact with other nuclear factors to form macromolecular complexes active in transcription. The possible relationship between these complexes and the subnuclear localization of the osteoblast transcriptional apparatus will also be discussed.

Key words: Osteoblast — Transcription — Runx2 — Regulation

General Concepts: The Role of Cooperativity in Transcription Factor Function

The regulatory regions of all eucaryotic genes have the following overall organization (for overview, see [1]). (1) A core promoter containing a polII preinitiation complex bound to TATA or related sequences on DNA from -50 to +50 from the transcription start site. (2) Regulatory promoter/enhancer/suppressor regions containing general and tissue-specific enhancer sequences, these can be from several dozen to thousands of base pairs away from the core promoter. (3) Enhancer binding proteins (general and tissue-specific) that are thought to function by cooperatively interacting with each other, and a separate group of proteins known as transcription accessory factors (TAFs). Enhancer-binding proteins and TAFs act cooperatively to bend DNA so that contact can be made with the general transcription apparatus of the core promoter. Another important function of TAFs is to remodel chromatin in the vicinity of the core promoter via modifications such as histone acetylation. This weakens histone:DNA contacts and allows relaxing of the nucleosomes to increase accessibility of the preinitiation complex to the DNA. In addition, TAFs may directly activate the preinitiation complex.

Implicit in understanding how tissue-specific enhancer binding proteins like Runx2 function is the recognition that each factor binds DNA with only weak affinity, but that combinations of factors can cooperatively enhance DNA interactions and interactions with other factors. A related concept is that subtle modifications of individual factors (including posttranslational modifications such as phosphorylation) can greatly affect transcription by affecting cooperativity. Viewing factors as functioning in complexes can also provide a model for how cell lineage is defined during differentiation. Thus, in the early stages of differentiation when a pluripotent stem cell makes initial lineage commitments, a specific factor might associate with all genes that are to be expressed in subsequent lineages. This factor by itself would not activate the final transcriptional program, but would be available to form complexes with additional lineage-specific factors later in the differentiation program. These complexes would then activate transcription via association with TAF-like factors. One prediction of this model is that individual lineage-specific transcription factors, while essential for expression of a specific phenotype, would not be expected to

Correspondence to: R. T. Franceschi; E-mail: rennyf@umich.edu

function in isolation. Instead, it is proposed that a hierarchy of factors specifies potential fates of cells as they progress down a particular lineage. Similarly, presence of a lineage-specific factor in the nucleus could also specify, via its ability to interact with other nuclear proteins, whether a cell would respond to a given external signal such as might be received from the extracellular matrix, hormones, or growth factors.

Specification of the Osteoblast Lineage Requires at Least Two Nuclear Factors

What is the evidence that this hierarchical control model can be used to explain how cells become committed to the osteoblast lineage? To date, only two bone-associated enhancer-binding proteins have been identified through gene deletion studies as being essential for osteoblast formation. These are Runx2, the osteoblast-specific product of the Cbfa1 gene [2], and the recently described zinc finger-containing transcription factor, Osterix (Osx) [3]. Current evidence supports the view that Runx2 is required for early commitment of mesenchymal cells to chondro/osteoblastic lineages as well as expression of phenotypic markers in differentiated cells whereas Osx is downstream of Runx2 and functions to further define the differentiation potential of chondro/osteogenic precursors toward the osteoblast phenotype [3, 4]. Consistent with this view, Runx2 is expressed in condensed chondrogenic mesenchyme as early as embryonic day 12.5, well before osteoblasts or hypertrophic chondrocytes appear [2]. Osx, in contrast, is absent from early chondrogenic cells, first appearing in differentiating chondrocytes and the perichondrium of E 13.5 embryos. At later times, its expression is restricted to osteogenic cells. Osx knockouts have no osteoblasts and do not express osteoblast marker genes, but their chondrocytes appear to undergo normal hypertrophy that is accompanied by mineralization of the growth plates and expression of a variety of hypertrophic chondrocyte markers [3]. Cbfa1 knockout mice also lack osteoblasts, but their chondrocytes do not undergo hypertrophy [5, 6]. Although we do not yet know whether Runx2 and Osx physically or functionally interact and/or co-localize to the same nuclear compartment, Osx is clearly necessary for Runx2 transcriptional activity *in vivo* since the putative Runx2 target genes osteocalcin, bone sialoprotein, and Collal are silent in Osx-/-animals , yet Runx2 is still expressed at normal levels [3]. Clearly, an important area for future studies will be to understand the relationship between these two factors in the control of gene expression in bone.

Runx2 is Regulated by Multiple Signal Transduction Cascades

Studies on the regulation of Runx2 transcriptional activity in bone provide a second example of how interactions between Runx2 and other nuclear factors may specify whether or not an osteoblast can respond to a particular external signal. Osteoblasts must respond to a variety of factors during bone formation and remodeling which provide information about the extracellular matrix environment as detected by integrin-extracellular matrix (ECM) interactions (i.e., Has an appropriate ECM been synthesized? Is the cell in contact with this ECM? Is the ECM experiencing mechanical loads?) and hormone/growth/differentiation factor levels in the extracellular mileau (i.e., Are the combined signals from endocrine/juxtacrine/autocrine factors telling the cell to grow or differentiate? Lay down new bone matrix or resorb existing matrix?). We propose that these responses require participation of Runx2 in osteoblast-associated signal transduction cascades that modify the level of Runx2 phosphorylation. This in turn modifies the ability of Runx2 to heterodimerize with other signaling pathway-specific nuclear factors to form transcriptionally active complexes. Two examples of Runx2 phosphorylation will be discussed; that induced by ECM-integrin binding and by fibroblast growth factor 2 (FGF2). We will then describe examples of heterodimer formation with members of the AP-1 and SMAD families of nuclear factors that are involved in regulation by parathyroid hormone (PTH) and the bone morphogenetic proteins (BMPs).

Activation of Runx2 by ECM and FGF2 Requires MAP Kinase-Dependent Phosphorylation

Differentiation of osteoblasts and induction of osteoblast-related genes requires secretion of a type I collagen-containing ECM. This matrix interacts with cells by binding to $\beta1$ subunit-containing integrins which then signal to the cell nucleus to regulate gene expression [7–9]. We showed that Runx2 mediates the response of preosteoblasts to matrix signals in that ECM-dependent induction of the osteocalcin (OCN) gene requires Runx2 and its cognate DNA binding site in the OCN promoter, osteoblast-specific element 2 (OSE2) [10]. This Runx2-dependent increase in transcriptional activity is not accompanied by a significant change in Runx2 mRNA or protein even though large increases are seen in the *in vitro* binding of Runx2 to OSE2 DNA, as measured by gel retardation assays [7, 10]. These results suggest that Runx2 is regulated by a posttranslational process.

To further explore the mechanism of Runx2 activation, we examined bone signal transduction pathways downstream of integrin activation. The MEK/ERK branch of the mitogen-activated protein kinase (MAPK) pathway provides a plausible link between cell surface integrin activation and subsequent stimulation of Runx2-dependent transcription. We recently showed that the potent MAPK pathway inhibitor, U0126, rapidly and specifically inhibits both ERK phosphorylation

and ECM-dependent induction of the OCN gene [11]. In addition, overexpression of a constitutively active form of MEK1, the kinase immediately before ERK 1/2 in the MAPK cascade, was also shown to induce OCN gene expression through a mechanism requiring Runx2 and an intact OSE2 sequence. Significantly, MEK1 transfection also increased Runx2 phosphorylation [12]. The C-terminal proline/serine/threonine (P/S/T)-rich domain of Runx2 was required for MAPK-dependent phosphorylation and regulation of transcriptional activity [13]. This 270 amino acid region is thought to be important for regulation of Runx2 activity [14] and, interestingly, also contains a 30 amino acid nuclear matrix targeting sequence [14].

Studies with FGF2 provide an additional example of how activation of the MAPK pathway can stimulate osteoblast transcription. This growth factor is an important *in vivo* regulator of skeletal development and growth [15]. Of particular interest, activating mutations in FGFR1 up-regulate Runx2, enhance differentiation of calvarial osteoblasts, and stimulate osteocalcin gene expression [16]. Since a major route for FGF receptor signaling involves activation of the MEK/ERK branch of the MAP kinase pathway [17], we examined whether FGF2 induction of the osteocalcin gene required MAPK activity and Runx2 phosphorylation [13]. Similar to the studies on ECM regulation, FGF2 rapidly induced the phosphorylation of both ERK and Runx2 and stimulated OCN gene transcription. Furthermore, these responses were completely blocked by MAPK inhibitors and required the P/S/T domain of Runx2.

Role of Runx2-Nuclear Protein Binding in the Response of Osteoblasts to PTH and BMPs

Runx2-related factors can physically interact with a number of nuclear proteins including transcriptional coactivators such as p300/CBP, Cbfβ which forms heterodimers with all members of the Cbfa family of factors, the transcriptional corepressor, TLE/Groucho and its antagonist, HES-1, the retinoblastoma tumor suppressor (Rb protein), SMADs and AP-1 proteins [18–22]. The formation of complexes between Runx2 and these factors provides a mechanism for generating an osteoblast-specific response to different signaling molecules (e.g., PTH signaling via AP-1 factors, BMP signaling via SMADs).

One early step in PTH-dependent bone remodeling is the PKA-dependent transcriptional activation of the collagenase 3 gene. This is mediated through the PTH receptor 1 which activates both PKA and PKC-related signal transduction pathways. Studies using the collagenase 3 promoter have been particularly informative for understanding how signals from different pathways can be integrated to regulate gene expression in bone.

Systematic analysis of the proximal promoter identified an 110 bp region as being essential for PTH responsiveness [23]. This region contains a Runx2 binding site at -133 bp from the transcription start site and an AP-1 site at -48 bp. Mutations in either site that abrogated the binding of Runx2 or c-Fos/c-Jun, respectively, totally abolished PTH stimulation of promoter activity (D'Alonzo, 2001 #1648) while hormonal responsiveness was enhanced by overexpression of both Runx2 and AP-1 binding proteins. These studies suggest that cooperative interactions take place between these factors. More direct evidence for this concept comes from immunoprecipitation experiments which demonstrated direct interactions between Runx2 and c-Fos/c-Jun [26, 27]. This interaction requires the runt domain of Runx2 and the leucine zipper domain of the two AP-1 factors. Evidence was also presented that PTH, via activation of the PKA pathway, can stimulate Runx2 phosphorylation [24].

Signaling by bone morphogenetic proteins (BMPs) provides a second example of how cooperative interactions between Runx2 and other nuclear proteins may regulate gene expression. Like other members of the TGF-β superfamily, BMPs regulate gene expression using specific SMAD proteins. BMP signaling uses the receptor-regulated SMADs 1, 5, and 8 (R-SMADs) and the common partner protein, SMAD4 [25]. R-SMAD-SMAD4 complexes regulate gene expression by binding to specific enhancer sequences on target genes (SMAD binding elements or SBEs). Since BMP treatment or overexpression of Runx2 have both been reported to induce osteoblast-specific gene expression in mesenchymal cells [2, 26] and previous studies had shown direct interactions between SMADs and Runx2 [21, 27], we examined whether these two factors could cooperatively interact to stimulate osteoblast differentiation. To specifically test for cooperativity between Runx2 and BMP signaling pathways, we examined effects of BMP on osteoblast gene expression in the presence or absence of Runx2 [13]. For these studies, recombinant adenoviruses engineered to express BMPs 2, 4 or 7 or Runx2 under the control of a CMV promoter [28] were used to transduce the pluripotent C3H10T1/2 mesenchymal cell line. Individual BMPs or combinations of BMPs were found to only modestly stimulate osteoblast differentiation as measured by induction of OCN, alkaline phosphatase, and mineralization. A slightly higher level of induction was observed in cells transduced with the Runx2 virus alone. In contrast, co-transduction of cells with optimal liters of BMP and Runx2 viruses synergistically increased differentiation to levels up to 10-fold greater than was seen with individual virus treatments. In separate studies, we showed that ECM-dependent activation of the MAPK pathway and Runx2 phosphorylation are also required for responsiveness of osteoblasts to BMPs [11]. This suggests that Runx2 must

be in the activated, phosphorylated state before it can interact with the BMP pathway. Although the molecular basis for these synergistic interactions is not established, an attractive model is that the observed increases in gene expression are explained by cooperative interactions between Runx2 and R-SMADs on the promoters of osteoblast-related genes.

Future Directions: Does Functional Cooperativity Require the Formation of Stable Subnuclear Complexes Between Runx2 and Other Nuclear Proteins?

The studies described in the preceding paragraphs show that Runx2 is necessary for both the commitment of mesenchymal cells to chondrogenic and osteogenic lineages as well as for responsiveness of osteoblasts to extracellular signals. This central role of Runx2 in bone cell function may be explained by its ability to functionally and physically interact with a number of nuclear factors and signal transduction cascades. The accompanying article by Drs. Stein and Lian describes the subnuclear localization of Runx2 to specific nuclear matrix sites. Evidence is presented that Runx2 can recruit other nuclear proteins such as TLE/Groucho and SMADs to these sites. In addition, it is proposed that this recruitment is essential for normal transcriptional regulation since a mouse line containing a premature termination codon in Runx2 that eliminated 152 amino acids of the C-terminal P/S/T domain, including the nuclear matrix targeting sequence, had a phenotype similar to the Cbfa1 null mutant (i.e., complete absence of osteoblasts or hypertrophic chondrocytes) [29]. Since this same region contains binding sites for several other nuclear factors such as TLE/Groucho, SMADS, and Rb [18–21] and is adjacent to putative phosphorylation sites [13], more specific mutations will be required to establish the importance of nuclear matrix targeting to Runx2 actions *in vivo*. Nevertheless, this study clearly shows that the P/S/T domain of Runx2 is critical for biological activity and further emphasizes the importance of interactions with other nuclear proteins in transcriptional control. Our studies show that Runx2 transcriptional activity is greatly stimulated by phosphorylation. We believe that the phosphorylation state may also control interactions between Runx2 and other nuclear proteins. Clearly, an important area for future investigation will be to determine whether there is a relationship between Runx2 phosphorylation and its ability to recruit other factors to nuclear matrix sites and the role this recruitment plays in the activation of osteoblast gene expression.

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