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Regulation of Gene Expression in Osteoblasts

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Abstract

In recent years, much progress has been made in understanding the factors that regulate the gene expression program that underlies the induction, proliferation, differentiation and maturation of osteoblasts. A large and growing number of transcription factors make important contributions to the precise control of osteoblast formation and function. It has become increasingly clear that these diverse transcription factors and the signals that regulate their activity cannot be viewed as discrete, separate signaling pathways. Rather, they form a highly interconnected, cooperative network that permits gene expression to be closely regulated. There has also been a substantial increase in our understanding of the mechanistic control of gene expression by co-factors such as acetyltransferases and histone deacetylases. The purpose of this review is to highlight recent progress in understanding the major transcription factors and associated epigenetic co-regulators involved in osteoblastogenesis and the mechanisms that determine their functions as regulators of gene expression.

Keywords

Runx2; Osterix; HDAC; Atf4; Lef1; NFATc; SMAD; miRs

Introduction

Osteoblasts produce extracellular matrix proteins and regulators of matrix mineralization during skeletal development, remodeling and regeneration. They arise from mesenchymal precursors that undergo a well-defined program of gene expression as they progress through osteoblastic commitment, proliferation, and terminal differentiation. Mature osteoblasts produce a characteristic extracellular collagenous matrix that subsequently becomes mineralized by deposition of hydroxyapatite crystals. In addition to protective and structural functions, mineralized bone is the major calcium and phosphorous reservoir and provides a supportive niche for hematopoiesis. Deregulation of bone formation and maintenance is an important component of common human diseases such as osteoporosis and arthritis, as well as numerous rare diseases and disorders, and is also involved in the pathology of metastatic bone disease that afflicts many cancer patients. Osteoblastic bone formation and repair underlies the healing of orthopedic injuries and of craniofacial and dental restorative procedures. Understanding the cellular and molecular mechanisms that drive osteoblast formation is thus of great importance for the development of better therapeutic options for these and other clinical conditions.

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The lineage commitment of multipotent mesenchymal cells is driven by the selective expression of so-called master transcriptional regulators. Thus, MyoD directs these cells into the myogenic pathway[1]; PPAR γ 2 promotes adipogenesis[2]; Sox9 drives chondrocyte development[3], and Runx2 is necessary for the osteoblast lineage[4,5]. Following lineage commitment, osteoprogenitors undergo a proliferative stage, characterized by the production of proteins such as histones, fibronectin, type I collagen c-Fos, c-Jun and p21 [6]. Subsequently, they exit mitosis, transition to expressing genes such as alkaline phosphatase, bone sialoprotein and type I collagen, as they commence to produce and mature an osteogenic extracellular matrix. Finally, they express genes involved in mineralization of the extracellular matrix such as osteocalcin, osteopontin and collagenase [7]. This highly regulated program of gene expression and cellular differentiation is governed by the expression and activity of transcription factors including Runx2, osterix, SMADs, TCF/LEF, NFATc1, Twist, AP-1, and ATF4. These factors do not act in isolation, but interact to integrate diverse signals and fine-tune gene expression. In addition to these transcription factors, recent work has discovered microRNAs as important regulators of osteoblast gene expression. The role of each of these factors as regulators of gene expression is presented and some notable recent findings are discussed below.

Runx2

Runx2 is frequently described as the master regulator of osteoblastogenesis [reviewed by 8]. It acts throughout the induction, proliferation, and maturation of osteoblasts and regulates expression of many osteoblast genes. Many of the signaling pathways and transcription factors that influence osteoblastogenesis do so by influencing the production or activity of Runx2. *RUNX2* haploinsufficiency causes cleidocranial dysplasia, a condition characterized by hypoplastic clavicles, various dental defects, delayed ossification of the skull bones and a number of other skeletal abnormalities, while homozygous mutation of *Runx2* in mice is lethal due to a complete lack of mineralized bone[4,5,9]. Runx2 expression is poorly correlated with expression of its target genes, indicating that Runx2's activity is regulated by additional factors. Indeed, Runx2 is subject to posttranslational regulation by phosphorylation, acetylation and ubiquitination. In addition to its Runt-class DNA binding motif, the Runx2 protein contains multiple domains that mediate either transcriptional activation or repression through associations with co-activators or co-repressors[10,11]. These many modes of control enable Runx2 to function as a master regulator, integrating diverse signals to activate or repress transcription in a precise spatio-temporal manner and in response to changing physiological needs.

Several recent papers shed light into the factors and mechanisms that control Runx2 function. Histone acetyltransferases, such as p300, CBP, PCAF, MOZ and MORF, are co-activators of Runx2[12,13]. They add acetyl groups to lysine residues of histone and non-histone target proteins, which modifies protein function by a variety of mechanisms including altered protein-protein interaction and altered protein stability. In the case of nucleosomal histones, acetylation is associated with a more open chromatin structure, recruitment of bromo-domain proteins, and increased transcriptional activity at a locus, while histone deacetylation, catalyzed by histone deacetylases (HDACs) is correlated with chromatin condensation and transcriptional repression. The interaction between Runx2 and HDACs was first suggested by the discovery that histone deacetylase inhibitors reduce the activities of some Runx2 repression domains [14]. A candidate gene approach was then used to show that HDAC6 binds Runx2 and represses its activity. Subsequent studies have shown that Runx2 binds and is functionally inhibited by HDAC3, HDAC4, HDAC5, and HDAC7[15–20]. Inhibition of HDACs with small molecule inhibitors or RNAi-mediated suppression accelerates osteoblast differentiation in vitro, suggesting that HDACs may be clinically relevant targets for bone anabolic therapies[15,17,21–23]. HDAC proteins are

known to form large multi-component repressive complexes composed of cofactors such as NCor, SMRT and Sin3a, as well as multiple HDACs. It remains poorly understood how these complexes participate in regulation of Runx2 activity, although it has been shown that Runx2 target gene expression is repressed by HDACs through multiple distinct mechanisms and in response to various osteogenic signals such as BMP2 and PTH (Figure 1). Lamour et al. showed that Runx2 recruits HDAC3 to the BSP promoter, where it represses transcription by deacetylating histones[20] (Fig. 1A). The Runx2 protein is subject to proteolysis in response to Smurf1 and Schnurri-WWP1 ubiquitin ligases[24–29]. Jeon et al. found that BMP2 protects Runx2 from Smurf1-catalyzed proteolysis by stimulating Runx2 acetylation through a SMAD-dependent mechanism [16]. Runx2 acetylation by p300 is counteracted by HDAC4 and HDAC5, which remove the acetyl groups from Runx2, thus promoting Runx2 ubiquitin-mediated proteolysis (Fig. 1B). Interestingly, estrogen receptor-related receptor γ , a orphan nuclear receptor whose expression in osteoblasts is stimulated by BMP2, competes with p300 for binding to Runx2 and inhibits BMP2-induced osteoblast formation[30]. Runx2 recruits both HDAC6[14] (Fig. 1D) and HDAC7[15] to chromatin; however, these factors utilize distinct and incompletely understood mechanisms to repress Runx2 target gene transcription. Inhibitors of deacetylase enzymatic activity attenuate repression by HDAC6 [14], whereas HDAC7 represses Runx2 through an unknown mechanism that does not require its deacetylase domain or catalytic activity[15]. BMP2 activates protein kinase D 1 (PKD1), which phosphorylates HDAC7, leading to a transient export of HDAC7 from the nucleus, and freeing Runx2 from HDAC7's repression[31](Fig. 1C). Despite considerable similarities between the HDAC4, HDAC5 and HDAC7 proteins, HDAC7 does not appear to significantly affect Runx2 protein levels (EDJ, unpublished observation). HDACs 4, 5 and 7 can be exported from the nucleus in response to the same set of protein kinases, yet they exhibit different subcellular distributions and respond differently to BMP2 stimulation in osteoblast-like cells[31]. Thus, despite the significant progress that has been made in determining the functional role of HDACs in osteoblasts, our understanding of their regulation remains incomplete.

Parathyroid hormone (PTH) is another important regulator of skeletal physiology that stimulates Runx2 interactions with acetyltransferases. PTH is a strong inducer of MMP-13 transcription in osteoblasts[32,33]. Stimulation of these cells with PTH leads to a protein kinase A-dependent binding of p300 to Runx2 on the MMP-13 promoter, resulting in increased histone acetylation and gene transcription[34]. PTH also regulates Runx2 activity through other mechanisms such as phosphorylation [35] and promoting interactions with AP-1 transcription factors [36,37]. Finally, PTH decreases Runx2 protein stability by ubiquitin-mediated proteolysis, limiting PTH stimulation of osteoblastic genes[29].

Osterix

Osterix (*Osx*, also known as *Sp7*) is a zinc finger transcription factor expressed in osteoblasts and, like Runx2, is required for bone formation[38]. *Osx*-null mice die at birth due to their lack of mineralized skeletons. Bones formed by intramembranous ossification are entirely non-mineralized, while endochondral bones exhibit regions of mineralized cartilage, indicating that *Osx* functions specifically in osteoblasts. Despite its evident importance in bone formation, relatively little is known about regulation of *Osx* expression, its functional partners, or its direct target genes. *Osx* expression was believed to be downstream of Runx2, because Runx2 expression is normal in *Osx*-null mice, while *Osx* expression is absent in Runx2-knockout mice[38]. This was confirmed through characterization of a Runx2-binding element in the *Osx* gene promoter[39]. Osterix activation of the Collagen IA1 promoter is enhanced by binding of NFATc1 to *Osx*, an interaction that is disrupted by calcineurin[40]. Another function of osterix is as an inhibitor of canonical Wnt signaling by inhibiting DNA binding of TCFs[41]. Despite these

interesting findings, the details concerning the regulation and function of *Osx* are incompletely understood.

ATF4

RSK2 is a serine/threonine kinase mutated in Coffin-Lowry Syndrome, a disorder that includes various skeletal abnormalities. The positive role of ATF4 on osteoblast formation was recognized with the findings that it is a substrate for the RSK2 kinase and *Atf4*-deficiency decreased bone formation[42], while forced accumulation of ATF4 induced osteoblastic gene expression in non-osseous cells[43]. ATF4 forms a complex with Runx2[44] at the osteocalcin promoter to increase osteocalcin transcription. Recent studies demonstrated that the transcriptional activity of this complex is furthered by PTH signaling[45] and by associations with C-EBP[46] and the TFIIA γ general transcription factor[47]. Interestingly, ATF4 in osteoblasts was recently found to regulate energy metabolism through decreased insulin production and insulin responsiveness via altered osteocalcin and leptin endocrine signaling pathways[48,49].

SMADs

The BMP and TGF β families of growth factors have long been recognized as vital regulators of skeletal physiology. TGF β or BMP signaling leads to phosphorylation and nuclear translocation of receptor-activated SMADs (rSMADs), which interact directly with DNA and associate with other transcription factors to regulate gene transcription. rSMADs direct mesenchymal cells into the osteoblast lineage through induction of Runx2 expression[50]. They also interact with the Runx2 protein to synergistically regulate transcription[50–54]. The SMAD-interaction domain in Runx2 has been identified and is continuous with the nuclear matrix targeting sequence, which is necessary for Runx2 function[53,55]. SMADs are inactivated by Smurf-directed ubiquitination, resulting in their proteolytic degradation. An interesting feedback loop between BMP/SMAD/Runx2 signaling is indicated by recent studies that showed that BMPs act through Runx2 to induce expression of SMAD6, an inhibitory SMAD protein that represses BMP signaling[56]. SMAD6 stimulates Runx2 ubiquitination and degradation by Smurf1[57]. This process would be a potential mechanism to prevent excess BMP/Runx2-mediated osteogenesis.

NFATc1/Calcineurin

NFATc1 is a transcription factor that plays a central role in osteoclast formation and in T-cell development[58]. In unstimulated cells, NFATc1 is highly phosphorylated and localized to the cytoplasm. Intracellular calcium signaling activates the phosphatase calcineurin, which dephosphorylates NFATc1, permitting its nuclear import and NFATc1-mediated gene expression. Given the importance of NFATc1 in osteoclastogenesis, it would be expected that administration of calcineurin inhibitors would suppress resorption and increase bone mass; however, calcineurin inhibitors actually result in osteopenia. Koga et al. resolved this paradox by showing that in addition to inhibiting osteoclasts, calcineurin inhibitors blocked osteoblast maturation and mineralization by preventing a previously unknown synergy between NFATc1 and osterix in osteoblasts[40]. In a subsequent study, Choo et al. showed that overexpressed constitutively active (nuclear) NFATc1 inhibited MC3T3 osteoblast differentiation in vitro and reduced expression of osteocalcin as a result of inhibited TCF/LEF transcriptional activity, which was due to sustained recruitment of HDAC3 and decreased histone acetylation at the proximal osteocalcin promoter[19].

Twist

Twist is a basic helix-loop-helix transcription factor that regulates differentiation of multiple cell types. Heterozygosity for Twist-1 in mice or humans results in premature fusion of the skull sutures, suggesting that Twist antagonizes osteoblast formation[59–61]. One mechanism through which Twist-1 acts to impair osteoblastogenesis is by binding to the Runx2 DNA binding domain and inhibiting its ability to bind DNA[62]. Twist also inhibits BMP/SMAD responsive transcription by forming a complex with Smad4 and HDAC1[63].

AP-1

The AP-1 class of transcription factors is composed of heterodimers of Fos -related factors (c-Fos, Fra1, Fra2 and FosB) and Jun proteins (c-Jun, JunB and JunD). Multiple Fos and Jun proteins are highly expressed in proliferating osteoprogenitors. Their expression decreases during differentiation such that Fra2 and JunD are the primary AP-1 components present in mature osteoblasts[64]. Targeted deletion and transgenic overexpression strategies have been used to examine the role of individual Fos and Jun proteins in mice. Deletion of c-Fos had little effect on bone formation[65], while its overexpression led to osteosarcomas[66]. Fra1 and Δ FosB (an alternative splice variant of FosB) overexpressing mice exhibit enhanced osteoblast formation[67,68], while deletion of Fra1 or JunB reduced bone mass. Recent work by Chang et al. demonstrates that inhibition of NF- κ B signaling specifically in differentiated osteoblasts promotes bone formation through increased Fra1 expression[69].

These observations indicate that AP-1 proteins promote bone formation. In contrast, deletion of JunD increased bone mass, apparently by increasing expression of Fra1, Fra2 and c-Jun, suggesting that JunD represses expression of other AP-1 proteins in osteoblasts[70]. A number of direct targets of AP-1 in osteoblasts have been identified, and include the osteocalcin, collagenase-3 (MMP13), bone sialoprotein, and alkaline phosphatase promoters[71]. At these promoters, AP-1 physically and functionally interacts with other transcription factors such as the vitamin D receptor and Runx2 to regulate gene expression. Yet another layer of complexity to AP-1 signaling involves alternative protein isoforms. As mentioned above, Δ FosB, which is a splice variant of FosB that lacks the amino-terminus, promotes osteoblast formation through incompletely understood mechanisms. Translational initiation of the Δ FosB mRNA from an internal methionine can produce a further truncated protein, known as Δ 2 Δ FosB, which lacks any known transcriptional activation domains, yet enhances osteoblast formation by increasing BMP/SMAD signaling[72].

Tcf7/Lef1 Transcription Factors

Tcf7 proteins and Lef1 are high mobility group proteins best known as nuclear effectors of canonical Wnt signaling. Activation of the canonical Wnt signal transduction pathway stabilizes β -catenin, which translocates to the nucleus and associates with Tcf/Lef1 transcription factors, displacing HDACs and other co-repressors while recruiting additional co-activators to stimulate gene expression[73–77]. Tcf7 (also known as Tcf1), Tcf7L2 (Tcf4) and Lef1 are expressed in osseous cells[78–81]. Although Tcfs are functionally redundant in some instances, emerging evidence demonstrates distinct roles for Tcf7/Lef1 factors in osteoblasts. Expression of a mutated and constitutively activated version of the Tcf7/Lef1 co-activator, β -catenin, in osteoblasts using the (2.3)Col1A promoter stimulated osteoprotegerin expression, leading to decreased osteoclastogenesis and bone resorption, but had little effect on osteoblast formation[78]. Conversely, Tcf7 knockout mice showed decreased OPG expression, enhanced osteoclast activity and increased resorption[78]. Lef1 also contributes to osteoblast function. Noh et al. showed that *Lef1*^{+/-} female mice exhibited reduced osteoblast activity resulting in decreased bone mass[82], while homozygous *Lef1*^{-/-} mice show reduced body size and die by two weeks of age[80]. Lef1 expression decreases

during osteoblast differentiation and overexpression of Lef1 inhibited differentiation and expression of late osteoblast markers, indicating that Lef1 inhibits late stages of osteoblastogenesis[79,83]. Subsequent work by Hoepfner et al. identified an alternative variant of Lef1, Lef1 Δ N, which lacks the N-terminal β -catenin binding domain[84]. Lef1 Δ N expression increases during differentiation and in response to BMP signaling and Runx2, and leads to accelerated osteoblast formation. Likewise, although Runx2 expression is directly enhanced through canonical Wnt signaling through TCF-7 [85], Runx2's transcriptional activity is repressed by binding to Tcf/Lef transcription factors in osteoblasts, providing a novel means for feedback between Wnts signaling and Runx2[79]. Wnt signaling is believed to act downstream of BMP signaling in the differentiation of pre-osteoblastic cells, as induction of osteoblasts by Wnt3a or activated β -catenin is independent of BMP signaling, whereas attenuated Wnt signaling impairs BMP2-induced expression[86–88]. Wnt-responsive transcription in osteoblasts is also antagonized by FGF signaling, through decreased expression of frizzles and TCF/LEFs[89]. Together, these studies demonstrate functional complexity within the TCF/LEF family and illustrate some of the opportunities for regulatory crosstalk to integrate diverse signals and modulate gene expression in osteoblasts.

Zinc Finger Proteins

Two major families of zinc-finger transcription factors are the Kruppel-like factors (KLFs) and Specificity Proteins (Sp). Members of both groups participate in regulation of gene expression in osteoblasts through interactions with other transcription factors at target gene promoters. Zfp521, a KLF protein, is expressed in osteoblast precursors, osteoblasts and osteocytes, as well as chondrocytes[90]. Its expression increases during osteoblast differentiation and in response to PTHrP, while BMP2 decreases ZFP521 levels. ZFP521 binds to Runx2 and antagonizes Runx2 gene transactivation, and overexpression of ZFP521 in *in vitro* osteoblast cultures impairs their differentiation. These observations indicate an inhibitory role for Zfp521 in osteoblasts. Unexpectedly, mice overexpressing ZFP521 in osteoblasts, under control of the OG2 osteocalcin promoter element, exhibit increased bone mass, even though isolated calvarial osteoblasts from these mice show impaired osteoblastic differentiation. The authors speculated that this difference may stem from the OG2 promoter not being expressed until relatively late in osteoblastogenesis. The Sp family of transcription factors is ubiquitously expressed (with the exception of Osx, which is also known as Sp7), and is involved with both basal and induced gene expression. Sp1 cooperates with ETS transcription factors at the Runx2 P1 promoter to stimulate transcription of Runx2[91]. In osteoblasts, Sp1 and Sp3 cooperate with TGF β -responsive SMADs to induce the β_5 integrin promoter[92]. Similarly, Sp1 cooperates with Runx2 to mediate PTH-induction of the matrix gla protein promoter, while Sp3 is an inhibitor of this promoter [93].

Regulation of osteoblast gene expression by microRNAs

An exciting new development in regulation of osteogenesis concerns the role of micro RNAs (miRs). These are short non-coding RNAs, ranging from 18–25 nucleotides, that regulate gene expression by binding to the 3'-UTR of mRNAs for specific target genes and inhibiting gene expression either by promoting degradation of the target mRNAs or inhibiting their translation[94]. Many of these miRs inhibit osteogenesis through repression of osteoblastic genes. In an important study, Li et al. identified a novel mechanism through which BMP-2 promotes osteoblastogenesis[95]. By RNA expression profiling, they identified a set of 22 miRs whose expression was reduced by BMP2 stimulation of C2C12 mesenchymal cells. These miRs are predicted to inhibit a range of pro-osteogenic factors; hence, reduced levels of these miRs should enhance expression of osteogenic genes. Osteoblastic proliferation is inhibited by miR-125b, which inhibits the ErbB2 receptor

tyrosine kinase [96]. Two miRs that inhibit expression of *Dlx5* in pre-osteoblasts have been identified[97]. miR-29a and miR-29c are expressed in response to canonical Wnt signaling and inhibit expression of the extracellular matrix protein Osteonectin, which is important in numerous processes in skeletal physiology[98]. Not all miRs are functional inhibitors of osteoblastogenesis. TGF β signaling inhibits osteogenesis, and MiR-210 acts as a positive regulator of osteoblastic differentiation by inhibiting expression of *AcvR1b*, a type I receptor for TGF β [99].

Concluding Remarks

In conclusion, a great deal of progress has been made in recent years towards the identification and characterization of the proteins that control gene expression in osteoblasts. Regulation of gene expression and protein activity is subject to coordinate control by diverse inputs. These discoveries provide exciting opportunities for development of new cell-based therapies and refinement of existing treatments for clinical manipulation of bone formation, maintenance, and regeneration. Novel gene regulatory mechanisms such as the microRNAs have uncovered new mechanisms for regulation of skeletal physiology, while the recognition of osteoblasts as endocrine cells that participate in systemic control of energy metabolism have brought renewed interest and significance into continued investigation of osteoblast gene expression.

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ABBREVIATIONS

AP-1	activator protein 1
ATF4	activating transcription factor 4
BMP	bone morphogenic protein
BSP	bone sialoprotein
CBP	CREB binding protein
C-EBP	CAAT-enhancer binding protein
FGF	fibroblast growth factor
HDAC	Histone deacetylase
KLF	Kruppel-like factor
LEF	Lymphoid Enhancer Factor
miR	micro-RNA
MMP-13	matrix metalloproteinase-13
NCoR	nuclear receptor co-repressor
NFATc1	nuclear factor of activated T-cells
OPG	osteoprotegerin
Osx	osterix
PCAF	p300/CBP associated factor
PKD1	protein kinase D 1

PTH	parathyroid hormone
RSK2	ribosomal S6 kinase 2
Sp	Specificity protein
TCF	T-cell factor
TGF-β	transforming growth factor β
Zfp	Zinc finger protein

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Figure 1. Interactions between Runx2, Histone Deacetylases and Acetyltransferases

(A) Runx2 recruits p300 or HDAC3 to target gene promoters, where they acetylate/deacetylate chromatin to regulate transcriptional activity. PTH promotes p300-Runx2 interactions and enhances gene expression. (B) BMP2 promotes acetylation and stabilization of Runx2 protein by p300, while HDAC4 and HDAC5 promote its deacetylation, ubiquitination, and proteolysis. (C) HDAC7 represses Runx2 through unknown means. BMP2 stimulates PKD1 to phosphorylate HDAC7, freeing Runx2 from HDAC7 repression. (D) Regulators of HDAC6-Runx2 interaction and the HDAC6 repressive mechanism have not been identified.