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Signaling and transcriptional regulation in osteoblast commitment and differentiation

Wei Huang[1], **Shuying Yang**[2,3], **Jianzhong Shao**[1], and **Yi-Ping Li**[1,2,3]

[1]College of Life Sciences, Zhejiang University, Hangzhou, China

[2]Department of Cytokine Biology, Forsyth Institute, Harvard School of Dental Medicine, Boston, Massachusetts

[3]Department of Developmental Biology, Harvard School of Dental Medicine, Boston, Massachusetts

Abstract

The major event that triggers osteogenesis is the transition of mesenchymal stem cells into bone forming, differentiating osteoblast cells. Osteoblast differentiation is the primary component of bone formation, exemplified by the synthesis, deposition and mineralization of extracellular matrix. Although not well understood, osteoblast differentiation from mesenchymal stem cells is a well-orchestrated process. Recent advances in molecular and genetic studies using gene targeting in mouse enable a better understanding of the multiple factors and signaling networks that control the differentiation process at a molecular level. Osteoblast commitment and differentiation are controlled by complex activities involving signal transduction and transcriptional regulation of gene expression. We review Wnt signaling pathway and Runx2 regulation network, which are critical for osteoblast differentiation. Many other factors and signaling pathways have been implicated in regulation of osteoblast differentiation in a network manner, such as the factors Osterix, ATF4, and SATB2 and the TGF-beta, Hedgehog, FGF, ephrin, and sympathetic signaling pathways. This review summarizes the recent advances in the studies of signaling transduction pathways and transcriptional regulation of osteoblast cell lineage commitment and differentiation. The knowledge of osteoblast commitment and differentiation should be applied towards the development of new diagnostic and therapeutic alternatives for human bone diseases.

Keywords

Osteoblast; Runx2; Osterix; ATF4; SATB2; Wnt signaling; TGF-Beta signaling; hedgehog signaling; fgf signaling; ephrin signaling; sympathetic signaling; Review

2. INTRODUCTION

Physiological bone turnover can be divided into 2 temporal phases: modeling, which occurs during development, and remodeling, a lifelong process involving tissue renewal. Remodeling starts with removal by osteoclasts of matrix, a mixture of insoluble proteins in which type I collagen is predominant (>90%) and a poorly crystalline, chemically modified hydroxyapatite. Following resorption, osteoblasts are recruited to the site, where they secrete and mineralize new matrix. The increased activity of osteoclasts caused by estrogen withdrawal causes bone loss and osteoporosis, a frequent low-bone mass disorder in

Send correspondence to: Dr. Yi-Ping Li, Department of Cytokine Biology, The Forsyth Institute, 140 The Fenway, Boston, MA 02115, Tel: 617-892-8260, Fax: 617-262 4021, ypli@forsyth.org.

postmenopausal women leading to structural instability and a high fracture risk. Estrogen deficiency is known to play a critical role in the development of osteoporosis, while calcium and vitamin D deficiencies and secondary hyperparathyroidism also contribute (1). Osteoporosis is a factor in more than 1.5 million fractures each year in the United States alone. Costs have been estimated at more than $17 billion a year, particularly from hip fractures, more than 75% of them in women. A better understanding of bone quality, coming from biochemical markers and refined imaging techniques, will help predict who is most at risk of debilitating fractures. One of the main approaches to gleaning details about the quality of bones is to measure the activity of osteoclasts and osteoblasts, the cells that remodel bone and thus influence its structural properties (2).

The osteopetrotic rodent models provided the first demonstration of contributions by osteoblasts to bone remodeling defects in osteopetrotic rats with specific genotypes (i.e., ia/ia, tl/tl, and op/op)(3–5). To understand the mechanisms of osteoblast differentiation, Ducy et al. initially studied the regulation of expression of Osteocalcin, the most osteoblast-specific gene (6). Over the past 12 years, molecular and genetic studies have modified our understanding of osteoblast differentiation (Table 1). Differentiation along the osteoblast lineage has been shown to depend on two transcriptional regulators. Runt-related transcription factor 2 (Runx2) and Osterix are both required for the early and late stages of osteoblast differentiation, whereby Runx2 is a master regulator that acts upstream of Osterix (7). Since the volume of Wnt literature is increasing rapidly, a few aspects of current interest have been selected here, mainly focused on Wnt signaling through its receptors (Frizzleds) to ß-catenin, which is often called the canonical pathway. The recent discoveries of signal transduction pathways and transcription factors critical for osteoblast differentiation and function have opened up new approaches to understanding the pathogenesis of osteoporosis. We focus this review on the process of osteoblast differentiation, mainly because this is the area in which much progress has recently been made.

3. OSTEOBLAST FUNCTION

Bone mass is regulated both by the number of mature osteoblasts and by their bone-forming activity. Osteoblasts are a bone-specific mesenchymal cell type, which is defined by its three functions. Firstly, it is responsible for bone formation, i.e., the synthesis and secretion of most proteins of the bone extracellular matrix (ECM), and also expresses genes that are necessary and sufficient to induce mineralization of this ECM. Osteoblasts deposit osteoid on the pre-existing mineralized matrix only. Osteoblasts ligate existing matrix via ß1 integrins, forming a monolayer that is linked by cadherins. Once active, the cells secrete a matrix containing type I collagen and smaller but significant amounts of osteocalcin, matrix gla protein (MGP), osteopontin, bone sialoprotein, many minor components, and, importantly, growth factors such as bone morphogenic proteins (BMPs) and transforming growth factor-ß (TGF-ß). Key ectoproteins, including progressive ankylosis gene (ANK) and tissue nonspecific alkaline phosphatase (TNAP), export pyrophosphate generated intracellularly and cleave this small-molecule inhibitor of calcification, respectively (8). Surprisingly, none of the genes involved in inducing ECM mineralization are osteoblast or even bone specific. However, osteoblast is the only cell type, along with odontoblasts and cementoblasts in teeth, in which they are coexpressed. A team led by Mary Schweitzer of North Carolina State University in Raleigh describes dinosaur blood vessels-still flexible and elastic after 68 million years-and apparently intact osteocytes (9). The unusual preservation of the originally organic matrix may be due in part to the dense mineralization of dinosaur bone, because a certain portion of the organic matrix within extant bone is intracrystalline and therefore extremely resistant to degradation. These factors, combined with as yet undetermined geochemical and environmental factors, presumably also contribute to the preservation of soft-tissue vessels.

In contrast to their proapoptotic action on osteoclasts, bisphosphonates increase the survival of osteoblastic cells (10). Despite its broad biological importance, the control of phosphate homeostasis remains incompletely understood. Most of the genes contributing to its normal regulation have been identified by studying genetic defects leading to different hypophosphatemic disorders. Dentin matrix protein 1 (DMP1) is a unique molecule that initiates osteoblast differentiation by transcription in the nucleus and orchestrates mineralized matrix formation extracellularly, at later stages of osteoblast maturation (11). Targeted ablation of both Dmp1 alleles in mice resulted in shorter bones and vertebrae, a highly expanded zone of proliferation and hypertrophic chondrocytes in the growth plate of younger mice and broad sclerotic long bones in older animals (12). These skeletal findings, which were initially thought to represent a form of chondrodysplasia, led to the conclusion that DMP1 is required for normal postnatal bone and tooth formation. Recently, however, decreased serum phosphate and calcium levels have been observed (13), demonstrating the similarity of this phenotype with different hypophosphatemia-induced forms of rickets. With the identification of DMP1 mutations as the cause of autosomal recessive hypophosphatemia (ARHP) (14), we have a better understanding of the regulation of phosphate homeostasis.

Secondly, osteoblasts are required for osteoclast differentiation, and thereby for bone resorption (15). The two main genes required for osteoclast differentiation, M-Csf and Rankl, are expressed in osteoblasts, and the requirement for osteoblasts to induce osteoclast differentiation can be bypassed by culturing osteoclast progenitor cells in the presence of M-CSF and RANKL. Recent studies by Elefteriou *et al.* provide interesting evidence that sympathetic signaling via ß2-adrenergic receptors increases expression of RANKL on osteoblast progenitor (16). Signaling by RANKL can be modulated by the decoy receptor Osteoprotegerin (Opg), which binds RANKL and is secreted from osteoblasts and several other cell types. Additionally, calcineurin/NFAT signaling in osteoblasts controls the expression of chemoattractants (CCL8, CCL6, and CCL12), which may recruit osteoclast precursors to bone and influence osteoclastogenesis (17).

Thirdly, bone and marrow are intrinsically linked with haematopoietic stem cells (HSCs), and their primitive progeny are located proximal to the endosteal surface of trabecular bone (18). Recently, bone morphogenetic protein receptor 1a (Bmpr1a) and parathyroid hormone (PTH)/PTH-related protein (PTHrP) receptor (PPR) studies identified osteoblasts as crucial cellular components of the HSC microenvironment and thus have defined a niche for HSCs (19, 20). These genetic studies provide mechanistic insights into osteoblast-mediated HSC expansion (21). The Bmpr1a studies identified a specific subset of N-cadherin-expressing osteoblasts that form an N-cadherin/ß-catenin adherens complex with HSCs, perhaps mediating the attachment or adhesion of HSCs within their niche (19). In the PPR studies, Notch signaling was implicated, because the Notch ligand Jagged 1 was highly expressed in osteoblasts and Notch-activated in HSCs (20). Subsequently, advanced imaging studies have demonstrated that the HSCs reside in close proximity to the bone-lining osteoblasts (22) as well as blood vessels, which may constitute an alternative niche (23).

The hematopoietic cytokine granulocyte-colony stimulating factor (G-CSF) is widely used clinically to elicit HSPC mobilization for life-saving BM transplantation. It has been postulated that G-CSF triggers the release of specific proteases, leading to the degradation of adhesion molecules and chemokines. In particular, CXCL12 or stromal-derived factor 1 (SDF-1) and its receptor CXCR4 have been implicated as a key ligand-receptor pair responsible for retention of HSCs in the bone marrow (24). However, the function of these proteases has been challenged by other data, indicating that G-CSF-induced mobilization was normal in mice lacking virtually all neutrophil serine protease activity, even when combined with a broad metalloproteinase inhibitor (25). This suggests that other proteases and/or other mechanisms are involved. Given that the ability to mobilize stem cells varies

between patients, these processes beg further investigation. The work of Katayama *et al.* takes a step into this void, providing a mechanism for G-CSF-induced mobilization of HSCs and suggesting that the sympathetic nervous system may regulate the egress of stem and progenitor cells from their niche (26). Bone marrow, bone, and the nervous system now appear to integrate signals to regulate HSCs. If hematopoiesis is any guide, niches may be nodal points where multiple, previously disconnected systems collide (27).

4. OSTEOBLAST ORIGIN AND CELL LINEAGE

Osteoblasts, which play central roles in bone formation, are derived from undifferentiated mesenchymal cells that also have the capacity to differentiate into chondrocytes, adipocytes, and myoblasts (28). There are three major stages of osteoblastogenesis: proliferation, matrix maturation, and mineralization, which are characterized by sequentially expressed distinctive osteoblast markers. The most frequently used markers of the osteoblast differentiation process are alkaline phosphatase (ALP), type I collagen (Col1a1), osteopontin (OPN), bone sialoprotein (BSP), osteocalcin (OCN), and PPR (Table 2). In general, ALP, BSP and Col1a1 are early markers of osteoblast differentiation, while PPR and OCN appear late, concomitantly with mineralization. OPN peaks twice, during proliferation and then again in the later stages of differentiation.

Following initial lineage commitment, a phase of lineage expansion ensues which culminates normally in permanent cell cycle withdrawal. The initial cell division is asymmetric, giving rise to another stem cell (self-renewal) and a committed osteoprogenitor. Following commitment, the stem cell gives rise to the transit-amplifying compartment (29). This phase is associated with intensive proliferative activity. The preosteoblast is an intermediate stage, which expresses both STRO1, ALP, PPR, and type I collagen, and is committed to the osteoblast lineage with extensive replicative capacity, but no self-renewal capacity (30). *In vitro*, the use of agents such as retinoic acid can induce further differentiation in the preosteoblast. The mature osteoblast expresses ALP, OPN, BSP, and OCN, and lies adjacent to newly synthesized osteoid (Table 2). This stage, which is responsible for the laying down of bone, has limited replicative potential (31). The cumulative effect of the recruitment of stem cells and their expansion, and the functional capacity of mature osteoblasts, is measured by rates of bone formation *in vivo*. The second key step initiates terminal differentiation and permanent cell cycle withdrawal. The terminal stage of the bone lineage is the post-mitotic osteocyte, often found isolated within bone, presumably embedded within advancing osteoids. As an alternate fate, a proportion of cells in the transient amplifying compartment may also terminate in apoptosis.

A range of cytokines modulate osteoblast differentiation, including bone matrix-derived TGF-ß, bone morphogenic protein 2 (BMP-2), BMP-4, and BMP-7, and their inhibitors noggin, chordin, gremlin, and sclerostin, the last identified by positional cloning of families with increased bone mass. Similarly, numerous hormones impact osteoblast function positively including IGF-1, PTH, PTHrP, 1,25(OH)2D3, leptin, glucocorticoids, the Notch pathway, and members of the leukemia inhibitory factor/IL-6 family.

5. OSTEOBLAST SIGNALING

Several signaling systems are known to play important roles during osteoblast development (Figure 1).

5.1 Wnt signaling pathway

5.1.1 Wnt signaling: an overview—Wnts, a family of secreted glycoproteins with multiple inhibitors, are ligands for the family of 7-membrane-spanning frizzled (FZD)

receptors. The Wnt family of secreted factors is involved in numerous aspects of cellular biology, ranging from cell fate determination, polarity and differentiation to migration, proliferation, and function. Wnt proteins are divided into two classes. The first class activates the canonical Wnt signaling pathway, which involves the formation of a complex between Wnt proteins, FZD, and low density lipoprotein (LDL) receptor-related protein 5 (LRP5) or LRP6 receptors (32, 33). The noncanonical Wnt5a class binds FZD proteins, activates heterotrimeric G proteins, and increases intracellular calcium via protein kinase C-dependent mechanisms or induces Rho- or c-Jun N-terminal kinase (JNK)-dependent changes in the actin cytoskeleton (34).

Although Wnt proteins signal through several pathways to regulate cell growth, differentiation, function, and death, the Wnt/ß-catenin or canonical pathway appears to be particularly important for bone biology (35, 36). The complexities of the Wnt/ß-catenin signaling pathway in multiple cell types have been reviewed elsewhere (37, 38), and an outline of the current model of Wnt signal transduction is shown in Figure 1. Wnt proteins released from or presented on the surface of signaling cells act on target cells by binding to the FZD/LRP5/6 complex at the cell surface. Signals are generated through the proteins Disheveled, Axin, and Frat-1, which disrupt the protein complex and inhibit the activity of glycogen synthase kinase 3 (GSK3), thus causing hypophosphorylation of its substrate, ß-catenin (39) (Figure 1). So, the “on state” involves increasing the post-translational stability of ß-catenin, through WNT-dependent inhibition of GSK3 (Figure 1). Stabilized ß-catenin then accumulates in the cytosol and translocates to the nucleus to activate target gene transcription. The most studied nuclear partners of ß-catenin are the lymphoid enhancer-binding factor/T cell factor (Lef/Tcf) transcription factor family (40, 41). ß-catenin displaces corepressors of Lef/Tcfs (e.g., Groucho, silencing mediator of retinoid and thyroid receptors and nuclear receptor corepressor [SMRT/NCoR]), and forms heterodimers with the Lef/Tcf proteins. With the assistance of transcriptional coactivators (e.g., p300 and cAMP response element-binding protein [p300/CBP]), this heterodimer binds DNA and initiates the transcription of target genes (42).

Extracellular WNT ligands can interact with a host of secreted antagonists, including the secreted FZD-related protein (sFRP) family and Wnt inhibitory factor 1 (WIF-1; Figure 1), preventing activation of the pathway. LRP5/6 coreceptor activity is inhibited by members of the SOST (Sclerosteosis gene product)(43) and Dickkopf (Dkk) families (44), all of which bind LRP5/6. Both Dkk1 and Dkk2 antagonize canonical Wnt signaling by simultaneously binding to LRP5/6 (45) and a single-transmembrane protein called kremen (44). Li *et al.* found ample evidence that Dkk2 has a role in osteoblast terminal differentiation and the effects of Dkk2 may not be entirely mediated by its Wnt signaling antagonistic activity (46). The precise mechanisms by which Dkk2 is involved in terminal osteoblast differentiation need further investigation.

If Wnts are not expressed or if their binding to receptors is inhibited, degradation of ß-catenin is facilitated via interactions with a protein complex consisting of adenomatous polyposis coli (APC), axin, and GSK3. APC and axin act as scaffold proteins allowing GSK3 to bind and phosphorylate ß-catenin, identifying it for degradation by the ß-TrCP-mediated ubiquitin/proteasome pathway. In the nucleus, prospective target genes of the pathway are kept in a repressed state by interacting with T-cell factor (TCF) and LEF transcription factors, with associated corepressors. So, in the “off state,” cells maintain low cytoplasmic and nuclear levels of ß-catenin, although ß-catenin is associated with cadherins at the plasma membrane, an association that spares it from the degradative pathway (Figure 1) (41). Despite the implied importance of canonical Wnt signaling in osteoblast biology, the role of the TCF/LEF family of transcription factors is unclear. Since ß-catenin participates in transcriptional complexes with molecules other than TCF/LEFs, some target genes may not

be regulated via TCF/LEF binding sites. For example, Kieslinger *et al*. found a strong synergy between LEF1 and EBF2, a member of the "early B cell factor"(EBF) family (47). Both EBF2 and LEF1/TCF proteins are expressed in specific cell types, and the functional synergy may contribute to a more restricted pattern of Wnt-regulated expression of Opg.

When considering WNT/ß-catenin signaling in disease, the potential involvement of multiple regulators of the effector ß-catenin must be included. Several components of the WNT/ß-catenin pathway might be regulated by WNT-independent processes. For example, there is evidence that integrin-linked kinase (ILK), a kinase that is regulated by integrin signaling, can inhibit GSK3, thereby stabilizing ß-catenin and activating ß-catenin target genes (48). Conversely, activation of the tumor suppressor p53 leads to degradation of ß-catenin (49). GSK3, as its name (glycogen synthase kinase) implies, is regulated by other pathways (50), raising questions about how signaling molecules are functionally compartmentalized. Finally, TGF-ß, Notch, and WNT pathways crosstalk in several contexts (34, 51, 52).

5.1.2 Wnt regulates osteoblastogenesis through the canonical pathway— Indisputably, Wnts are involved in embryonic skeletal patterning, fetal skeletal development, and adult skeletal remodeling (53–55). Unraveling the function(s) of Wnt proteins in the regulation of skeletogenesis has been a knotty problem, however, confounded by questions of functional redundancy, multiple times and sites of action, and the presence of other molecules that compete with Wnt function. The first indication that Wnt signaling plays a critical role in bone formation came from human studies where mutations in the Wnt coreceptor LRP5 are causally linked to alterations in human bone mass (56–59). These findings were supported by the observation that LRP5−/− mice also have low bone mass (60). Furthermore, gain-of-function mutations in LRP5 that increase Wnt signaling result in higher bone density in humans and mice (58, 61). Consistent with the effects of LRP5 on bone mass being mediated through canonical Wnt signaling, activation of this pathway *in vitro* results in the expression of alkaline phosphatase, an early osteoblast marker (62, 63).

One of the mechanisms whereby Wnt signaling increases bone formation is via stimulation of the development of osteoblasts, and there is considerable *in vitro* evidence supporting a role for Wnt/ß-catenin (i.e., canonical) signaling in this process (64–66). Higher levels of ß-catenin enhance bone formation with concomitant increases in expression of osteoblast-specific genes (64, 67), whereas conditional knockdown of the ß-catenin gene at an early developmental stage causes ectopic chondrogenesis and abnormal osteoblast differentiation (67–69). Clement-Lacroix *et al*. have shown that the GSK-3ß inhibitor LiCl increases bone formation in LRP5−/− mice (70).

Despite all of these data, the functions of Wnt signaling in the programs of bone biology remain unclear. Take, for example, two studies that show Wnt proteins inhibit the ability of human mesenchymal stem cells to differentiate into osteoblasts (71, 72) while three other studies show that Wnt signaling, through ß-catenin, contributes to osteoblast differentiation (35, 62, 73). The reason(s) for this apparent conflict are not immediately obvious. Most recently, Hill, T. P., *et al*; Day, T. F., *et al*; Glass, D. A., *et al* and Hu, H., *et al* provide compelling evidence that Wnt signaling represents both a cell-autonomous mechanism for inducing osteoblastic and suppressing chondrocytic differentiation in early osteochondroprogenitors and a mechanism in fully differentiated osteoblasts for stimulating the production of OPG, an inhibitor of osteoclast formation(67, 68, 74, 75).

5.1.3 Role of ß-catenin at various stages of osteoblast development—Among evolutionarily conserved signaling pathways, the pleiotropic effects of Wnt/ß-catenin signaling functions are well established in biological processes including embryogenesis,

tumorigenesis, and stem cell biology (68, 76). Recent experiments examining the conditional inactivation of ß-catenin in skeletal progenitors and using different Cre lines revealed that ß-catenin activity is essential for the differentiation of mature osteoblasts and, consequently, for bone formation in endrochondral bones (the long bones of the limbs) and membranous bones (in the skull) (67, 68, 75). These variable results likely arise because Wnt/ß-catenin signaling regulates bone development and accrual through different mechanisms at different stages of life (77). This concept is supported by the results of studies using mouse models in which targeted deletion of ß-catenin occurs early or late in osteoblastogenesis.

Perichondrial and periosteal cells failed to express the osteoblast commitment factor, Osterix, and acquired a chondrogenic fate (67, 68). Similar to the long bones, the osteoblastic progenitors differentiated in the absence of ß-catenin into chondrocytes (67, 68). These findings were substantiated by *in vitro* deletion of ß-catenin activity in dissociated calvarial cells. It is likely that ß-catenin activity is required in a bipotential precursor of the osteoblast lineage, the so-called osteochondroprogenitor, and indeed its absence steers the fate of mesenchymal precursors toward chondrogenesis (67, 68). As Runx2, but not osterix, is expressed in ß-catenin−/− mesenchymal cells (68, 75), ß-catenin seems to be required for osteoblast differentiation at the preosteoblast stage (Table 1). Further, ß-catenin/TCF1 enhances Runx2 expression and Runx2 promoter activity (78). By contrast, for differentiation into the chondrocyte lineage, ß-catenin levels must be low (Figure 2) (67, 68, 75).

Recently, a novel role for canonical Wnt signaling in postnatal bone homeostasis has been discovered by inactivating ß-catenin function in more mature osteoblasts using a Col1a1- and an OCN-Cre line (69, 74). Mice deficient in ß-catenin develop osteopenia. By contrast, activation of ß-catenin function in osteoblasts using the Col1a1- and the OCN-Cre line in combination with a conditional ß-catenin gain-of-function allele and a conditional APC allele, respectively, resulted in increased bone mass (69, 74). These mice manifest an osteopetrotic phenotype; however, no change in osteoblast activity or histomorphometric evidence of bone formation was observed. The altered bone resorption was caused by deregulation of Opg, a major inhibitor of osteoclast differentiation (74). Consistent with these observations in mice, autosomal-dominant osteopetrosis type I patients with a gain-of-function T253I mutation in LRP5 have decreased numbers of small osteoclasts, although osteoclastogenesis in response to RANKL was normal *in vitro* (79). Opg is a direct target gene of the ß-catenin-TCF complex in osteoblasts and Tcf1 is probably the relevant transcription factor required for Opg regulation; nevertheless, a possible role for Tcf4 cannot be excluded (65, 74). These mice demonstrate that ß-catenin regulates osteoclastogenesis through effects on expression of osteoprotegerin and RANKL (69).

5.2 TGF-beta Signaling

Once activated, TGF-ß can interact with its receptor to induce signaling. All members of the TGF-ß superfamily signal through a dual receptor system of type I and type II transmembrane serine/threonine kinases. The mothers against decapentaplegic (Smad) signaling turned out to play a central role in the transmission of signals from all receptors activated by the TGF-ß superfamily members to target genes in the nucleus.

Several members of the TGF-ß superfamily, such as BMPs, have potent osteogenic effects. BMPs are a group of phylogenetically conserved signaling molecules, and were initially identified by their capacity to induce endochondral bone formation (80–82). BMP-1 through BMP-7 are expressed in skeletal tissue, and BMP-2, -4 and -6 are the most readily detectable BMPs in osteoblast cultures (80, 83). BMPs are unique because they are implicated in the specification of both chondrocytes and osteoblasts (84), as well as in the subsequent

modification of the osteogenic program, where some BMPs promote bone formation, such as BMP-2, BMP-7, BMP-6 and BMP-9 (85), although Bmp3 acts as a negative regulator of bone formation (86).

On receptor activation, BMPs transmit signals through Smad-dependent and Smad-independent pathways, including ERK, JNK, and p38 MAP kinase (MAPK) pathways (87). Smads are the major signal transducers for the serine/threonine kinase receptors (88). There are three classes of Smads: 1) receptor-regulated Smads (R-Smads) that can be BMP activated, such as Smad 1, 5 and 8 (referred to as BR-Smads in this article), or TGF-ß activated, such as Smad 2 and 3 (TR-Smads); 2) common partner BMP and TGF-ß mediator Smads (Co-Smads), such as Smad 4; and 3) inhibitory Smads (I-Smads), such as Smad 6 and 7. Upon ligand stimulation and activation by type II receptors, type I receptors phosphorylate R-Smads, which in turn form complexes with Co-Smads (89) (Figure 1). The R-Smad/Co-Smad complexes then translocate into the nucleus and regulate transcription of target genes by interacting with various transcription factors and transcriptional co-activators or co-repressors. The third class of Smads, I-Smads, negatively regulates signaling by the R-Smads and Co-Smads. Runx2 and BR-Smads physically interact with each other upon activation of BMP signaling, and co-operatively regulate the transcription of target genes, leading to osteoblast differentiation of mesenchymal progenitor cells (90–92). BMP induces Runx2 expression in mesenchymal progenitor cells through the action of BR-Smads (93), and BR-Smads in turn interact with Runx2 and further induce osteoblastic differentiation. BMP does not directly induce the expression of Runx2 in mesenchymal cells (94), but it facilitates expression of Dlx5 in osteoblasts (95, 96), and Dlx5 then induces expression of Runx2 in osteoprogenitor cells.

Osteoprogenitor cells, e.g. C2C12 cells, have been widely used for the identification of BMP target genes during osteoblastic differentiation. Hey1 (also termed HesR1 and Herp2) and Tcf7 are transcription factors specifically expressed in osteoblast cells by BMP-2 treatment, and are involved in Notch and Wnt signaling, respectively (97). Using constitutively active BMP type I receptors, Korchynskyi *et al*. identified several genes as targets of BMP receptors in C2C12 cells, including transcription factors Hey1, ITF-2, and ICSBP (98).

Although the Smads are critical mediators in the TGF-ß signaling pathway, a substantial body of evidence illustrates the existence of additional, Smad-independent pathways. BMP-2 can activate ERK, JNK and p38 in osteoblastic cells and provide evidences that these MAP kinases have distinct roles in regulating alkaline phosphatase and osteocalcin expression (99, 100). Recent reports suggest that during osteoblast differentiation, BMP-2 activates JNK and p38 via protein kinase D (PKD), independent of protein kinase C (PKC) activity (101). It has been demonstrated that following TGF-ß and BMP induction, both the Smad and p38 MAPK pathways converge at the Runx2 gene to control mesenchymal precursor cell differentiation (102). Runx2 plays a central role in the BMP-2-induced trans-differentiation of C2C12 cells at an early restriction point by diverting them from the myogenic pathway to the osteogenic pathway (94, 103). It has been found that the homeobox gene Dlx5 is an upstream target of BMP-2 signaling and that it plays a pivotal role in stimulating the downstream osteogenic master transcription factor Runx2 (96). In turn, Runx2 acts simultaneously or sequentially to induce the expression of bone-specific genes that represent BMP-2-induced osteogenic trans-differentiation. However, inhibition of BMP signaling was shown to disrupt the ability of RUNX2 to stimulate osteoblast differentiation and transactivate an osteocalcin gene promoter-luciferase reporter in C3H10T1/2 cells (104). In conclusion, we can state that the JNK, ERK, and p38 MAPK pathways contribute considerably to the whole of TGF-ß-induced responses, but further characterization is needed to assess their importance in relation to the Smad-dependent and other TGF-ß-induced signaling pathways.

BMP-2 has been reported to induce Osterix (Osx) expression in mouse progenitor cells and chondrocytes (7, 105). Moreover, BMP-2-induced Osx expression is mediated by Dlx5 but is independent of Runx2 (106). In the bone microenvironment BMPs act in conjunction with other growth factors. Celil *et al.* identified the involvement of BMP-2 and IGF-I in mediating Osx expression in human mesenchymal stem cells (hMSCs) (107). The BMP-2-induced effect on Osx expression was mediated via p38 but not via Erk. Under osteogenic culture conditions, both Erk and p38 were involved in mediating Osx expression (107).

In the past several years, ubiquitin-mediated proteasomal degradation has been implicated in the regulation of BMP-2 and TGF-ß signaling pathways in various cell types (108, 109). Recently, Dupont, S. *et al* and Yamashita, M.*et al* highlighted the importance of this mechanism in regulating the *in vivo* effects of TGF-ß (110, 111). Dupont, S. *et al* redefined the role of a previously identified Smad1 ubiquitin ligase, Smurf-1 The absence of Smurf-1 causes the accumulation of MEKK2, resulting in activation of JNK, an event that is both necessary and sufficient for BMP sensitization in osteoblasts (110). Yamashita, M.*et al* identified and characterized a novel Smad4 ubiquitin ligase, Ectodermin (Ecto) and provided convincing evidence that Ecto represents the elusive determinant of ectoderm formation, acting as a critical inhibitor of all Smad-dependent TGF-ß signaling during vertebrate development (111).

5.3 Hedgehog signaling

Osteoblast progenitors can first be identified within the inner perichondrium adjacent to, and coincident with, the first appearance of hypertrophic chondrocytes. This tight linkage reflects a crucial role for Indian hedgehog (Ihh) signaling (112). Ihh is produced by prehypertrophic chondrocytes and appears to act directly on perichondrially located osteoblast progenitors to specify the osteoblast precursors (113, 114). The failure of activation of Runx2, a crucial early determinant of the osteoblast lineage, indicates that hedgehog (Hh) signaling acts to initiate an osteogenic program (115). Furthermore, Hh activates osteoblast development in a variety of mesenchymal and skeletogenic cell types *in vitro* (114, 116, 117). Genetic manipulation of Smo, which encodes an obligatory component of the Hh signaling pathway, has revealed that cells devoid of Smo, hence Hh signaling, fail to undergo osteoblast differentiation(114). Although Ihh signaling plays the crucial role in regulating the temporal and spatial program of early osteoblast commitment, Ihh doesn't play an on-going role beyond this stage(118). When smoothened (Smo) activity is removed in Osx1+ osteoblast precursors, normal bone secreting OcHigh osteoblasts are generated, and the endochondral skeleton at birth is indistinguishable from wild type(118). Whether this is also true in the adult is currently under investigation.

The interaction between Hh and Wnt signaling is probably complex. It has been demonstrated that nuclear localization of ß-catenin as well as expression of target genes for the Wnt canonical pathway were abolished in the perichondrium in Ihh−/− embryos (75). This could, among other possibilities, be due to the downregulation of Wnt expression in the absence of Hh signaling. Indeed, expression of Wnt9a and Wnt7b was either reduced or abolished in the perichondrium in Ihh−/− embryos. In addition, both genes were induced by Hh signaling in C3H10T1/2 cells (Figure 1). Alternatively, the Hh and Wnt signaling pathways could intersect intracellularly via common regulators such as Suppressor of fused [Su(fu)](119) and GSK3 (120, 121). Other pathways in addition to canonical Wnt signaling also contribute to Hh-induced osteogenesis. Of note, some groups reported that Hh-induced osteogenesis in C3H10T1/2 cells required BMP signaling (122, 123).

5.4 FGF signaling

The fibroblast growth factors (FGFs) are a family of secreted polypeptides that act through four related tyrosine kinase receptors (Fgfr1–Fgfr4) to regulate a plethora of developmental processes, and they are critical for the control of endochondral and intramembranous ossification (124).

Human diseases that manifest the precocious osseous obliteration of sutures, known as craniosynostosis, often result from gain-of-function mutations in FGF receptors 1–3 (Figure 1) (125, 126). Fgfrs 1–3 are expressed in the developing and mature skeleton in patterns suggestive of both unique and redundant function (124). In the developing growth plate, both Fgfr1 and Fgfr2 are expressed in condensing mesenchyme that will give rise to cartilage. Fgfr2 remains expressed in reserve chondrocytes and appears to be down regulated in proliferating chondrocytes, whereas Fgfr1 is expressed in hypertrophic chondrocytes. Later in development, Fgfr1 and Fgfr2 are both expressed in the perichondrium and periosteum, tissues that give rise to osteoblasts and cortical bone. In contrast to Fgfr1 and Fgfr2, Fgfr3 is prominently expressed in proliferating chondrocytes where it regulates cell growth and differentiation (127) and in differentiated osteoblasts where it regulates bone density and cortical thickness (128, 129). Mutations in Fgfrs account for many of the craniosynostosis and chondrodysplasia syndromes in humans (124, 130, 131).

Embryos lacking Fgfr1 (Fgfr1−/−) die shortly after gastrulation (132), necessitating a conditional knockout approach to address function later in development. Hypomorphic alleles of Fgfr1 or conditional inactivation of Fgfr1 prior to limb bud initiation affects digital patterning and the formation of some skeletal elements (133–135). Fgfr1 signaling in the osteoprogenitor cell normally acts to stimulate differentiation whereas it functions to suppress differentiation in differentiated osteoblasts. Thus, Fgfr1 signaling has stage-specific effects on osteoblast maturation (136).

The FGF ligands that signal to Fgfr1 in osteoblasts are not known; however, three FGFs (FGFs 2, 9 and 18) are likely candidates for this role. FGF9 and FGF18 are expressed in the perichondrium/periosteum, and mice lacking these FGFs show delayed ossification during mid-gestation skeletogenesis (137–139). FGF2 is expressed in periosteal cells and osteoblasts (140, 141) and adult FGF2−/− mice showed a loss of trabecular bone volume; however, no skeletal dysmorphology was reported in neonatal FGF2−/− mice (142). These observations suggest that FGFs 2, 9 and 18 may act alone or redundantly to regulate osteoblast activity and physiology and that FGFs 9 and 18 may constitute the predominant signals during embryonic development, whereas FGF2 may be more important during postnatal stages. Consistent with a role for FGF2 in more differentiated osteoblasts, bone marrow stromal cultures from FGF2−/− mice showed a significantly decreased ability to mineralize *in vitro* (142). It has been demonstrated that Runx2 is phosphorylated and activated by FGF2 via the MAPK pathway and suggests that FGF2 plays an important role in regulation of Runx2 function and bone formation (143).

Fgfr2−/− mice die at embryonic day 10.5 (E10.5), prior to skeletal development (144). The contribution of Fgfr2 signaling to skeletal development has been clarified to some extent by using splice form-specific knockouts and conditional gene deletion approaches in mice. These studies demonstrated that Fgfr2 positively regulates bone growth and the anabolic function of osteoblasts. The resulting phenotype of mice lacking mesenchymal Fgfr2 included skeletal dwarfism, decreased bone density, incomplete formation of the dorsal vertebrae and tarsal joint fusion (145, 146). Alternative splicing of Fgfr2 is tissue specific, resulting in epithelial variants (b splice forms) and mesenchymal variants (c splice forms) (147–149). Ligand binding studies demonstrate that mesenchymally expressed ligands such as FGF7 and 10, activate Fgfr2b, whereas FGF2, 4, 6, 8 and 9 activate Fgfr2c (150, 151).

As adults, Fgfr3−/− mice were osteopenic, suggesting a role for Fgfr3 signaling in differentiated, Fgfr3-expressing osteoblasts (128). Mice lacking either FGF18 or Fgfr3 exhibited expanded zones of proliferating and hypertrophic chondrocytes and increased chondrocyte proliferation, differentiation, and Indian hedgehog signaling. These data suggest that FGF18 acts as a physiological ligand for Fgfr3 (138). In addition, FGF18−/− mice had decreased endochondral and intramembranous bone formation suggesting that FGF18 positively regulates osteogenesis and or osteoblast function independent of Fgfr3 (138).

Two lines of evidence indicate that En1 regulates signaling mediated by Fgfrs. First, the activation ERK, normally restricted to the mature endosteal osteoblasts of wild-type calvarial bone, is severely impeded in En1 mutants. Second, En1 ablation results in loss of the FGF target gene Spry2 in ectoperiosteal osteoblasts. Furthermore, En1 may regulate alternative FGF-signaling effectors known to affect osteoblast differentiation, such as p38 MAPK or PKC (152–154). A precise temporal and spatial delineation of these intracellular pathways will enable a better understanding of how osteoblastic differentiation is coordinated by En1 and FGFs.

To study the effects of growth factors on hMSC, Kratchmarova *et al.* tested the effects of epidermal growth factor (EGF), platelet-derived growth factor (PDGF), FGF, and nerve growth factor (NGF) on cellular responses and observed that EGF and PDGF elicited the strongest responses (155). They also found that the differentiation of human mesenchymal stem cells into boneforming cells is stimulated by EGF but not PDGF. Mass spectrometry-based proteomics analysis demonstrated that more than 90% of these signaling proteins were used by both ligands, whereas the phosphatidylinositol 3-kinase (PI3K) pathway was exclusively activated by PDGF, implicating it as a possible control point. Indeed, chemical inhibition of PI3K in PDGF-stimulated cells removed the differential effect of the two growth factors, bestowing full differentiation effects onto PDGF.

5.5 Ephrin signaling

Ephrins have the capacity for bidirectional signaling. That is, when a cell expressing an ephrin receptor contacts a cell expressing an ephrin ligand, signals are transduced into both the ephrin receptor-expressing cell (forward signaling) and the ephrin ligand-expressing cell (reverse signaling). There are two classes of ephrins, the B class (ephrin B1 to B3) are ligands for EphB tyrosine kinase receptors (B1 to B6), whereas class A ephrins (A1 to A5) are ligands for GPI-anchored EphA receptors (A1 to A10) (156). In bone biology, ephrinB and EphB receptors control patterning of the developing skeleton (157), and disruption of ephrin signaling is implicated in a syndrome called craniofrontonasal syndrome (CFNS [MIM 304110]) (158). Zhao *et al.* now suggested that ephrin signaling is critical to the two-way communication between osteoclasts and osteoblasts (159). This bidirectional signaling is mediated by the transmembrane ephrinB2 ligand in osteoclasts and EphB4, a tyrosine kinase receptor, in osteoblasts (Figure 1). Using osteoblast-osteoclast co-culture assays, as well as loss- and gain-of-function studies *in vitro*, the authors demonstrated that reverse signaling from EphB4 in osteoblasts to ephrinB2 in osteoclast progenitors leads to the inhibition of osteoclast differentiation. On the other hand, EphB4 expression is constitutive and the forward signaling through EphB4 induces osteogenic regulatory factors, such as Dlx5, Osx, and Runx2, in calvarial osteoblasts, suggesting that EphB4 is at the top of the regulatory cascade during osteoblast differentiation. Zhao *et al.* demonstrated that forward signaling between the extracellular domains of ephrinB2 and EphB4 in osteoblasts stimulates their differentiation, a process that may be dependent on RhoA inactivation in osteoblasts. Expressing active RhoA in osteoblasts may block the ability of ephrinB to promote osteoblast differentiation. By contrast, McBeath *et al.*, using human mesenchymal stem cells, suggest that active RhoA enhances osteoblast differentiation (Figure 1) (160).

Therefore, the involvement of RhoA in EphB4 forward signaling will need to be confirmed by future pharmacological or genetic studies. This study establishes the concept that ephrin-Eph signaling contributes to bone homeostasis.

5.6 Sympathetic signaling

Neural control of bone metabolism, both trophic and atrophic, has been suggested by numerous experimental and clinical observations. Osteoblasts have been reported to express receptors for several neuropeptides, suggesting that they could indeed integrate multiple neuronal signals (161). Immunolabeling studies have revealed a close association between glutamate-, catecholamine-, or peptide-containing nerve fibers and osteoblasts or osteoclasts in the endosteum (162). Blockade of glutamate receptors was reported to reduce the DNA binding activity and expression of Runx2 in cultured osteoblasts (163). The effect of the sympathetic nervous system (SNS) on bone formation has only recently been elucidated using genetic models (164). These studies revealed that leptin induced bone loss through SNS-derived signals originating in the ventromedial hypothalamic nuclei (162).

Fu *et al* indicated that an important, new regulator of bone remodeling is the circadian cycle (165). The model that emerges from the results of Fu *et al*. suggests that signaling by ß2-adrenergic receptors first activates the transcription factor CREB (Figure 1). CREB in turn stimulates expression of clock genes, which mediate the antiproliferative function by inhibiting G1 cyclin expression, and AP1 genes, which stimulate proliferation of osteoblasts. The Fu *et al*.'s work provided evidence that the inhibition of osteoblast proliferation by clock proteins is the dominant effect. Pharmacological or genetic ablation of adrenergic neurotransmission indicates that norepinephrine (NE) signaling controls G-CSF-induced osteoblast suppression (26). Based on studies describing leptin-mediated neuronal control of osteoblast function (16, 162) and the fact that leptin and G-CSF receptors display a high degree of homology (166), it has been proposed that G-CSF signals directly in the hypothalamus through the leptin receptor (26).

Sequence-specific DNA-binding proteins are frequently encoded by gene families. Such proteins display highly conserved DNA-binding properties, yet are assumed to retain promoter selectivity. Yamamoto *et al*. addressed the factor-specificity issue with his update on the varied roles of the glucocorticoid receptor (GR) (167). They presented evidence that two ligands, the small nuclear hormone and the larger DNA molecule, act to direct the activities of GR. Most fascinating were the data showing that different hormone ligands could affect promoter selectivity (167).

6. OSTEOBLAST GENE TRANSCIRPATION: TRANSCRIPTION FACTORS THAT REGULATE OSTEOBLAST DIFFERENTIATION

Transcription factors that regulate osteoblasts include a range of homeodomain proteins: the activator protein (AP) family members Jun, Fos, and Fra, Smads, CCAAT/enhancer binding protein ß (C/EBPß) and C/EBPd, lymphoid-enhancing factor (a Wnt effector), twist, activating transcription factor 4, Runx2, and osterix, the last 3 of which are considered master genes for osteoblast differentiation (Figure 2).

Commitment of mesenchymal stem cells (MSCs) to tissue-specific cell types is orchestrated by transcriptional regulators that serve as “master switches.” A central regulator of bone formation is the Runx2 transcription factor which fulfills its role as a master regulatory switch through unique properties for mediating the temporal activation and/or repression of cell growth and phenotypic genes as osteoblasts progress through stages of differentiation (168). Though Runx2 is essential for osteoblast differentiation, this differentiation program also requires other genes, such as osterix, which encodes a transcription factor genetically

"downstream" of Runx2 (15). Thus, multiple genes regulate Runx2 activity and the effectiveness of Runx2 in stimulating osteoblast formation. It is perhaps not surprising then that Runx2 expression in osteoblast precursors predates by several days the first evidence for osteoblast activity.

6.1 Runx2

Runx2 is a member of the Runx (Runt-related factors) family of transcription factors (previously known as the acute myeloid leukemia [AML] factor, polyomavirus enhancer binding protein 2 [PEBP2], and core binding factor [CBF]). The family members, Runx1 (also called PEBP2aB, CBFA2 and AML1), Runx2 (also called PEBP2aA, CBFA1 and AML3), and Runx3 (also called PEBP2aC, CBFA3 and AML2), are encoded by distinct unlinked genes but share a common DNA recognition motif (TGTGGT) and heterodimerize with the ubiquitous subunit CBFß for stable DNA binding(169). Their highly conserved DNA binding domain is homologous to that from the Drosophila segmentation gene runt(170). In addition to the Runx DNA binding domain, Runx2 contains an active transactivation domain, rich in glutamine and alanine residues, and activates the Osteocalcin and Col1a1 genes(171, 172). Thus, Runx2 is an initial marker of the osteogenic cell lineage.

Runx2 is expressed in the thymus and testes (in T-lymphocytes tendon), and abundantly expressed in calcified cartilage and bone tissues, and is absent from the brain, heart, lung, gut, or liver (173–175). The function of Runx2 in bone formation has been demonstrated by analyzing its role in regulating the expression of the principal osteoblast-specific genes and by studying Runx2 null mice (171, 173, 176). Targeted disruption of Runx2 results in the complete lack of bone formation by osteoblasts, revealing that Runx2 is essential for both endochondral and membranous bone formation (Table 1) (173). The haploinsufficiency of the Runx2 gene, which leads to cleidocranial dysplasia, a genetic disease in humans that is characterized by hypoplastic clavicles, large open spaces between the frontal and parietal bones of the skull, and other skeletal dysplasias, is caused by heterozygous mutations in the Runx2 gene (177). Moreover, Runx2 is sufficient to induce osteoblast differentiation. This is true in cell culture, where forced expression of Runx2 in skin fibroblasts leads to osteoblast-specific gene expression (171), and *in vivo*, since ectopic expression of Runx2 leads to endochondral ossification in parts of the skeleton that would normally never ossify (178, 179). Importantly, Runx2 may function as an inhibitor of proliferation of progenitors, thus providing a mechanism for regulating the transition from growth to a postproliferative stage as a component of cellular commitment to the osteogenic lineage (180). Thus, Runx2 may be expressed in early osteoprogenitors to induce a program of gene expression required for lineage determination and differentiation of mesenchymal cells (Figure 2). Finally, Runx2 is also required for osteoblast function beyond differentiation (181, 182). These functions, along with its role during hypertrophic chondrocyte differentiation and vascular invasion, identify Runx2 as the most pleiotropic regulator of skeletogenesis (15). The literature now embraces the concept that Runx2 functions as a scaffold for the interaction with coregulatory proteins at subnuclear foci to provide an architectural basis for accommodating the requirements of biological control.

6.1.1 Runx2 upstream proteins—Msx2, which encodes a homeobox-containing transcription factor, is expressed in osteoblasts during development. The role of Msx2 during skull formation was first uncovered by human genetic studies. Indeed, one syndrome characterized by increased bone formation around the cranial suture, Boston-type craniosynostosis, is caused by an activating mutation in MSX2 (183). Msx2 inactivation in mice causes a marked delay of ossification in the bones of the skull and an overall decrease in bone volume. This phenotype is accompanied by a downregulation of Runx2 expression, indicating that Msx2 directly or indirectly regulates Runx2 expression (184). Recently,

Cheng *et al.*(185) and Ichida *et al.*(186) reported that a homeobox gene, the Msx2 gene, stimulates the commitment of mesenchymal cells into an osteoblast lineage in association with inhibition of adipogenesis (Figure 2). Bapx1, another homeobox protein encoding gene, is required for axial skeleton formation. In Bapx1-deficient mice Runx2 expression is downregulated in the axial skeleton (187), suggesting that Bapx1 is another activator of Runx2 expression. Beside defects in branchial arch patterning, Hoxa2−/− mice show an upregulation of the cartilage- and bone- specifying genes Sox9 and Runx2 (188).

Twist-1 (previously called Twist) and Twist-2 (previously called Dermo-1) encode vertebrate basic helix-loop-helix transcription factors homologous to Drosophila Twist, a mediator of dorsal-ventral patterning and mesoderm formation. Knockout of Twist-1 in mice leads to lethality at E10.5 due to failure of neural tube closure (189). Twist-1 heterozygotes (both in mice and in humans) exhibit craniosynostosis, a disease caused by premature osteoblast differentiation in the skull. Bialek *et al.* show that Runx2-induced osteoblast gene expression only occurs when expression of Twist genes disappears in osteoblast precursors (190). Twist-1 heterozygosity reverses skull abnormalities in Runx2+/− mice and Twist-2−/− reverses clavicular abnormalities in Runx2+/− mice and accelerates osteoblast differentiation in bones formed through endochondral bone formation. Twist proteins' antiosteogenic function is mediated by a novel domain, the Twist box, which interacts with the Runx2 DNA binding domain to inhibit its function. They conclude that Twist-1 and Twist-2 regulate the developmental action of Runx2 in bone formation through the direct interaction of these proteins. Bialek *et al.*, by bringing together the actions of Twist and Runx proteins, have clarified an important stage in bone formation and set a research agenda for the future (191).

It has long been recognized that the p53 tumor suppressor plays a pivotal role in preventing cancer. Two independent studies (192, 193) have addressed the role of p53 in bone differentiation in mouse models. In one case, Wang *et al.* examined skeletal structure and bone metabolism in p53 knockout mice (193). Conversely, Lengner *et al.* analyzed the effects of hyperactive p53 on bone formation caused by the conditional deletion of Mdm2 in osteoblasts (192). Surprisingly, and in contrast to the *in vitro* studies (194), both groups came to the same conclusion that p53 suppresses differentiation. Specifically, p53−/− osteoblasts displayed a marked propensity to differentiate, which was manifested by a modest but significant increase in bone formation and bone density in adult p53 knockout mice. Consistent with these results, the conditional deletion of Mdm2 in osteoblasts interfered with terminal differentiation, leading to late stage embryonic lethality, where the embryos displayed more porous and shorter bones. These findings suggest that the interplay between p53 and Mdm2 could either positively or negatively impact bone development. The studies of both Lengner *et al.* and Wang *et al.* provide compelling evidence that p53 suppresses osteoblast differentiation by repressing the expression of either Runx2 or Osterix (Figure 2) (192, 193). The subtle discrepancy that exists between the two studies (whether Runx2 or Osterix is the target of p53 action) may be related to how p53 activity is targeted and whether this mechanism alters the stage of cell differentiation. In either case, the concept that the absence of a tumor suppressor gene can enhance cell proliferation while favoring the differentiation of mesenchymal stem cells is intriguing but counterintuitive. It is likely that p53-deficient osteoprogenitors can still exit the cell cycle upon terminal differentiation, which may be enhanced as a result of the elevated expression of Runx2 and Osterix. These findings clearly establish p53 as a negative regulator of osteoblast differentiation both *in vitro* and *in vivo*.

Schnurri-3 (Shn3), a large zinc finger protein, was originally identified as a DNA binding protein of the heptameric recombination signal sequence required for V(D)J recombination of immunoglobulin genes (195); however, it also functions as an adapter protein in the

immune system (196). Jones *et al.* found that mice lacking Shn3 display adult-onset osteosclerosis with increased bone mass due to augmented osteoblast activity (197). Shn3 was found to control protein levels of Runx2 by promoting its degradation through recruitment of the E3 ubiquitin ligase WWP1 to Runx2. By this means, Runx2-mediated extracellular matrix mineralization was antagonized, revealing an essential role for Shn3 as a central regulator of postnatal bone mass (197). This area of osteoblast biology is still in its infancy, and the transcription factors that act upstream of Runx2 to control its expression remain to be identified.

6.1.2 Runx2 interacting proteins

6.1.2.1 Co-activators of Runx2: Runx2 protein is shown to interact with a number of transcriptional co-activators (Figure 2). The most important coregulatory protein, essential for enhancement of Runx DNA binding is Cbfß (also known as PEBP2ß), the non-DNA-binding partner of all three Runx proteins. Inactivation of Cbfß causes embryo lethality in mice between E11.5 and E13.5. This results from hemorrhaging in several tissues and the absence of liver hematopoiesis because Cbfß is a heterodimerizing partner of Runx1 and Runx3, which are essential for haematopoiesis. The timing of embryonic lethality precludes examining the role of the Cbfß subunit in osteoblast differentiation. Transgenic rescue of embryonic lethal Cbfß-null mice and `knock-in' of Cbfß fused in-frame to a cDNA encoding green fluorescent protein (198) resulted in mice that exhibited delayed ossification, indicating a role for Cbfß in bone. However, unlike Runx2-null mice that completely lack bone and osteoblasts, ossification is initiated in these mice, suggesting that Runx2 can act in the absence of Cbfß. Similar observations were made simultaneously by two other groups (199, 200). Runx2 also interacts physically and/or functionally with other well-characterized coactivators, including p300, CBP, MOZ, and MORF. p300 and CBP physically interact with various R-Smads upon ligand stimulation and enhance Smad-dependent transcription of target genes (201, 202). Neither p300 nor CBP has been coprecipitated with Runx2. Two members of the MYST family of HATs, MOZ and MORF, however, do interact with the activation domain of Runx2 and enhance activation of the osteocalcin promoter (203). This interaction may be functionally relevant to intramembranous bone formation, as mice with a mutation in the MYST gene have craniofacial defects (204). Grg5, pRb, and TAZ are other proteins that enhance Runx2-mediated transactivation (205–208). Although most Grg/TLE proteins are corepressors, Grg5 appears to be a dominantnegative form of longer Grg/TLE proteins, and thereby enhances Runx2 activity *in vivo* (207). The 14-3-3-binding protein, TAZ (transcriptional coactivator with PDZ-binding motif), coactivates Runx2-dependent gene transcription while repressing PPARg-dependent gene transcription, indicating that TAZ functions as a molecular rheostat to fine-tune the balance between osteoblast and adipocyte development (208).

6.1.2.2 Co-repressors of Runx2: Histone deacetylases (HDACs) remove acetyl groups from lysine residues on many proteins, including histones. The elimination of the acetyl group alters chromatin structure by removing a mark needed to recruit co-activating proteins and by facilitating chromatin condensation to promote transcriptional repression (209). General HDAC inhibitors, such as trichostatin A, increase Runx2-mediated activation, and Runx2 associates with several HDACs, including HDAC3, HDAC4, and HDAC6 (Figure 2) (210–212). HDAC3 interacts with the N terminus of Runx2. Suppression of HDAC3 expression in differentiating MC3T3-E1 cells accelerates matrix mineralization and the expression of bone marker genes such as osteopontin, bone sialoprotein, and osteocalcin (211). Vega *et al.* claimed that HDAC4 inhibits Runx2 activity by blocking Runx2 DNA binding (212). HDAC4-null mice display premature ossification due to early onset chondrocyte hypertrophy, and overexpression of HDAC4 inhibits chondrocyte hypertrophy, suggesting that Runx2 activity is controlled by HDAC4 in prehypertrophic chondrocytes

(212). HDAC6 was identified as a Runx2 binding protein in co-immunoprecipitation experiments designed to identify co-repressors that bind to the potent C terminus repression domain of Runx2 (210). Moreover, several other groups have reported that HDAC inhibitors increased Runx2-dependent activation of the osteocalcin promoter (211) and osteoblast maturation and differentiation (213, 214). However, results from a new study support a requirement for HDAC4 and -5 deacetylase activity to regulate acetylation, abundance and activity of Runx2 (215). As the phenotypes of other HDAC mutations are probed in detail, it will be exciting to learn whether these proteins have a general role controlling osteoblast differentiation.

Grg/TLE proteins are coexpressed with Runx2 in skeletal cells (216). The induction of the OCN gene correlates with downregulation of the level of Grg/TLE in mice skeletal tissues between E14 and birth and Grg/TLEs were shown to inhibit Runx2 dependent activation of OCN gene transcription (217). However, Grg5, a dominant negative form of long Grg/TLE proteins, can enhance Runx2 transcriptional activity *in vitro* (207). Depletion of Grg5 alone, with normal activity of Runx2, causes postnatal growth retardation in about 50% of the mice, and in Grg5 null Runx2+/− mice, the lack of Grg5 function combined with the heterozygous loss of Runx2 activity resulted in a growth deficiency which was more pronounced than would have been expected. This finding suggests that Grg5 and Runx2 interact with each other *in vivo* and that their combined activity is necessary for the activation of another factor important for bone and cartilage development. It is highly probable that the factor regulated by Grg5-Runx2 interaction is Ihh (207). Runx2 also interacts with YAP, a mediator of Src/Yes signaling, in the cytoplasm and translocates it to the nuclear matrix where YAP represses Runx2-mediated activation of the osteocalcin promoter (218). It is not yet known whether Runx2 associates with multiple corepressor complexes, or whether all of the corepressors mentioned above are components of the same complex.

One of the mechanisms by which transcription factors are regulated is by modulation of degradation. The proteosome degradation pathway decreases Runx2 protein levels to slow osteoblast differentiation (219). The ubiquitin-protein isopeptide ligase (E3) Smurf1 induces Runx2 degradation (220) and Smad6 enhances Smurf1-induced Runx2 degradation (221). For example, TNFa attenuates osteoblast differentiation by promoting Runx2 proteasomal degradation through up-regulation of Smurf1 and Smurf2 expression (222). Transgenic overexpression of Smurf1 in murine osteoblasts suppresses their differentiation and bone formation, while Smurf1-deficient mice develop age-dependent increases in bone mass (111, 223). HDAC4 and HDAC5 deacetylate Runx2, allowing the protein to undergo Smurf-mediated degradation (215).

6.1.3 Transcription factor partners of Runx2—Many transcription factors involved in regulation of the osteoblast differentiation process exert their action by interacting with Runx2 (Figure 2). Some provide costimulatory signals, while others directly repress Runx2 function by affecting its DNA binding activity and/or transactivation potential. DNA binding proteins that interact and cooperate with Runx2 to activate gene expression include AP1 (c-Fos and c-Jun)(224), BMP-responsive Smads (Smad1 and Smad5)(94, 225, 226), Ets1 (227), C/EBPß and -d (228, 229), Dlx5 (230, 231), Hes1 (232), and Menin (233) (Table 1). Most of these proteins interact with either the DNA binding domain or the activation domain of Runx2, although the binding sites for some have not been defined. It is generally believed that these transcription factors cooperate with Runx2 to facilitate the recruitment of coactivators and the assembly of higher-order transactivation complexes. Some proteins, including Hes1, may perturb TLE-Runx interaction both by competing with TLE corepressors for the binding site on Runx2 and by titrating TLE away from Runx2 (232). Furthermore, there is increasing evidence that Dlx5 promotes activation of Osteocalcin by

forming heterodimers with Msx2, which antagonizes the Msx2-mediated repression of Osteocalcin (230, 234). Other cooperating transcription factors, such as AP1, Smad1, and Smad5, integrate Runx2 with cell signaling pathways and to the extracellular environment (235, 236). Mutations that affect Runx2-Smad interactions are found in CCD patients and inhibit the ability of Runx2 to induce osteoblast differentiation after BMP stimulation (90).

Transcription factors that inhibit Runx2-dependent activation in mesenchymal cells or osteoblasts include Dlx3 (231), Lef1 (237), Msx2 (230), PPAR? (238), Smad3 (239), Hey1 (240), and Stat1 (241). These proteins repress Runx2 via several mechanisms, including binding the Runt domain and preventing DNA binding (e.g., Lef1, PPAR?) (237, 238), sequestering Runx2 in the cytoplasm (e.g., C/EBPd, Stat1) (241, 242), or unknown mechanisms that involve binding in or around the nuclear matrix targeting domain of Runx2 (e.g., Dlx3) (231).

6.2 Osterix

The discovery of a BMP2-inducible gene, Osx, a Kruppel-like Sp-1 binding factor, identified a second transcriptional regulator for the final stages of bone tissue formation (7). Osx contains a DNA-binding domain consisting of three C2H2-type zinc fingers at its C terminus that share a high degree of sequence identity with similar motifs in Spl, Sp3, and Sp4. In addition, Osx also contains a proline- and serine-rich transactivation domain and activates the OCN and Col1a1 genes. In Osx-null mutant mice, no endochondral or intramembranous bone formation occurs (Table 1) (7). The mesenchymal cells in Osx-null mutant mice do not deposit bone matrix, and cells in the periosteum and the condensed mesenchyme of membranous skeletal elements cannot differentiate into osteoblasts, although these cells express normal levels of Runx2. Interestingly, Osx-null osteoblast precursors in the periosteum of membranous bones express chondrocyte markers, such as Sox9 and Col2a1, suggesting that Runx2-expressing preosteoblasts are still bipotential cells and Osx acts downstream of Runx2 to induce osteoblastic differentiation in bipotential osteochondroprogenitor cells (Figure 2) (7). Currently, there is no evidence to indicate whether Runx2 and Osx functionally or physically interact. Milona *et al.* have reported that there is an OSE2 element in the Specificity protein-7 (Sp7; the human homologue of the mouse Osterix gene) regulatory region, so the Osx promoter may be a direct target of Runx2 (243). Further analysis on the relationship between Runx2 and Osx will be the focus of future studies.

Several studies implicate the presence of Runx2-independent mechanisms for ossification (60, 185). These studies implicate that additional signaling pathways may act in parallel to, or independent of, Runx2 during osteoblast differentiation. It has been shown that MAPK and PKD signaling pathways serve as points of convergence for mediating the BMP-2- and IGF-I-induced effects on Osx expression in mesenchymal stem cells (244). Additionally, Runx2 was required but not sufficient for the BMP-2-mediated Osx induction. This result indicated that additional factors (e.g. Dlx5) acting downstream of BMP-2 could induce Osx independent of the levels of Runx2 activity. Mechanistic analyses of the effect of FK506 on bone mass showed that NFAT cooperates with Osterix and accelerates osteoblast differentiation and bone formation(245). Overexpression of NFATc1 stimulates Osterix-dependent activation of the Col1a1 promoter, but not Runx2-dependent activation of the Bglap1 (encoding osteocalcin) promoter.

En1 expression temporally precedes that of the osteogenic determinant Osx, and, in the absence of En1, the onset of Osx expression is delayed. As Osx is necessary for potentiating the osteogenic fate of the skeletogenic mesenchyme (7), its perturbed expression provides a mechanistic basis for the delayed calvarial ossification in En1−/− mice (246). Furthermore, that Osx expression remains impaired in En1-null osteoblasts, suggests that En1 also lies

upstream of Osx during later phases of calvarial osteogenesis, and thus mediates distinct functions in osteoblast differentiation. En1−/− osteoblasts were deficient in mediating osteoid mineralization and exhibited reduced ALP activity, an enzyme that is essential for this process (8, 247). Moreover, ablation of En1 results in impaired Ocn and Bsp expression, genes that are normally associated with advanced osteoblast differentiation. Ocn expression has also been shown to be dependent on Osx (7).

6.3 ATF4

Using a combination of human genetic information, analysis of mutant mouse strains, and molecular studies, Yang *et al.* identify activating transcription factor (ATF4) as a critical substrate of RSK2 that is required for the timely onset of osteoblast differentiation, for terminal differentiation of osteoblasts, and for Bsp, and Osteocalcin expression (Figure 2) (248). Additionally, RSK2 and ATF4 posttranscriptionally regulate the synthesis of type I collagen, the main constituent of the bone matrix. These findings identify ATF4 as a critical regulator of osteoblast differentiation and function, and indicate that lack of ATF4 phosphorylation by RSK2 may contribute to the skeletal phenotype of Coffin-Lowry Syndrome (CLS) (Table 1) (248). In addition, treatment of non-osteoblastic cells with MG115, a proteasome inhibitor, induced ATF4 accumulation and resulted in activation of an Osteocalcin promoter luciferase construct as well as expression of endogenous Osteocalcin, a molecular marker of differentiated osteoblasts (249). This study establishes that ATF4, like other osteoblast differentiation factors, such as Runx2 and Osterix, has the ability to induce osteoblast-specific gene expression in non-osteoblastic cells.

Cooperative interactions between activating transcription factor 4 and Runx2/Cbfa1 stimulate osteoblast-specific osteocalcin gene expression (250).

6.4 SATB2

The patterning of skeletal elements and bone formation are generally thought to represent distinct pathways; however, evidence is emerging for crosstalk between these processes. This is illustrated in studies that establish the functional role of Hoxa2 in skeletal development, an inhibitor of bone formation and regulator of branchial arch patterning (188, 251). Understanding the mechanisms that mediate these dual roles of Hoxa2 will provide valuable insight into coordination of pathways governing bone patterning and differentiation (252). Grosschedl and colleagues make an important stride toward this goal by demonstrating that the nuclear matrix protein Satb2 represses Hoxa2 expression and is an activator of multiple steps of Runx2-dependent osteoblast differentiation (Figure 2) (253).

SATB2 is a recently cloned member of the family of special AT-rich binding proteins that binds to nuclear matrix-attachment regions (MARs) and activates transcription in a MAR-dependent manner (254, 255). In humans, translocations that involve the chromosomal region 2q32–q33 and are associated with a cleft palate under conditions of haploinsufficiency have been found to interrupt the SATB2 gene (256). Using targeted mutagenesis of Satb2 in mice, Dobreva *et al.* provide insight into how the nuclear matrix, chromatin remodeling, and gene activation come together to regulate osteoblast differentiation in development (253). The most striking phenotypes detected in mice lacking Satb2 are craniofacial defects in skeletal elements and the inhibition of normal osteoblast differentiation (Table 1). By combining an impressive series of molecular and genetic approaches, the authors reveal that SATB2 represses Hoxa2 expression in osteoblasts through direct recognition of a MAR-like sequence (Figure 2). Chromatin immunoprecipitation (ChIP) and transactivation experiments reveal that Satb2 can bind to and regulate Bsp and Ocn genes, themselves critical components in osteoblast formation. This strongly suggests that Satb2 has multiple inputs into transcriptional control of

osteoblast differentiation (Figure 2). On the basis of genetic synergy between mouse mutants, protein interaction and transactivation analyses, Dobreva *et al.* discovered that SATB2 directly interacts with and enhances the activity of both Runx2 and ATF4 (253). The interaction of SATB2 with ATF4 and Runx2 augment their binding to the cognate DNA-recognition elements, although SATB2 does not bind itself to the OSE1 and OSE2 sequences. SATB2 has been shown to recognize specific sequences, termed MAR sequences (254), and indeed, SATB2 was found to regulate the Bsp promoter by binding to a site that resembles a bona fide MAR element. Therefore, SATB2 can act not only as an activating or repressing DNA bound protein but also as a protein scaffold that enhances the activity of other DNA binding proteins. By its ability to regulate the expression or activity of multiple key determinants of skeletal development, SATB2 appears to represent a molecular node of a transcriptional network underlying this process (253). Distinguishing between the different modes of Satb2 activity will be important for a detailed understanding of how the nuclear matrix, chromatin structure, and transcriptional activity coordinate the regulation of multiple steps during osteoblast differentiation (252).

7. SUMMARY AND FUTURE DIRECTIONS

Formation of skeletal elements during embryogenesis and the dynamic remodeling of bone in the adult involve an exquisite interplay of developmental cues, signaling proteins, transcription factors, and their coregulatory proteins that support differentiation of osteogenic lineage cells from the initial mesenchymal progenitor cell to the mature osteocyte in mineralized connective tissue (168). The canonical pathway is an enticing target for developing drugs to battle skeletal diseases as Wnt/ß-catenin signaling is composed of a series of molecular interactions that offer potential places for pharmacological intervention (77). Several unresolved issues that continue to perplex us are offered here in the hope that this will stimulate their resolution. There is considerable evidence to show that Wnt signaling can cooperate in unexpected ways with other pathways. The functional interaction of Wnt signaling with other pathways probably accounts for the observation that Wnt/ß-catenin signaling regulates distinct sets of genes in different cells. The mechanisms by which this combinatorial signaling occurs, and whether it is functionally significant in the diseases and potential therapies, are largely unknown.

Although we have learned much in recent years regarding the role of canonical Wnt signaling in bone formation, some important questions remain to be addressed. For example, we know that most Wnts and FZDs are expressed in bone, but is there a role for Wnt and FZD specificity in the control of osteoblast physiology? The canonical pathway is clearly important for the regulation of bone formation, but do noncanonical pathways also play a role in bone metabolism? Finally, canonical Wnt signaling appears to control osteoclastogenesis through actions on osteoblasts, but do Wnts also have direct effects on bone resorbing cells? These and other questions are likely to be answered in the coming years.

Runx2 is essential for mesenchymal condensation, osteoblast development, and osteoblast maturation. Like all developmentally important proteins, multiple mechanisms actively control Runx2 activity. Since it is currently unknown how Runx2 is regulated during the development of the skeletal system, further studies are required to clarify the detailed mechanism of the temporal and spatial regulation of Runx2 for the normal development and homeostatic regulation of the skeletal system. Additional proteins will be added to the list of Runx2-interacting proteins in the next decade. Mechanisms regulating expression and subsequent activity of Osx, ATF, and SATB2 at different stages of osteoblast maturation are only beginning to be understood. Developmental regulation of ATF and SATB2 and cofactor interactions, as well as the possibility that interactions, especially those with other

transcription factors, may be either stimulatory or inhibitory depending on the promoter/enhancer context, are important considerations. Finally, insights into the mechanisms by which proteins and developmental signals affect these transcription factors' subcellular localization to the nuclear matrix will be needed to understand how they regulate gene expression.

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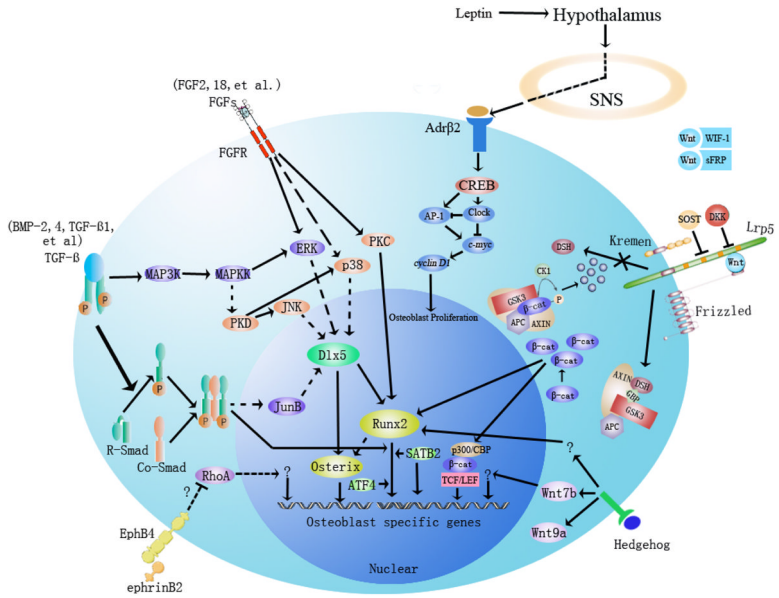

Figure 1.

Six important signaling networks of osteoblast differentiation. Binding of Wnt to the FZD receptor induces ß-catenin accumulation, which translocates to the nucleus to activate target gene transcription. Several transcription factors have been found crucial for osteoblast differentiation downstream of this signaling pathway, such as Runx2, Osterix, and ATF4. They are essential for differentiation of mesenchymal stem cells into differentiated osteoblasts and also function in the transcription of osteoclast-specific genes. The dotted lines indicate that the physiological function or the stage at which the factor mainly works remains to be proven.

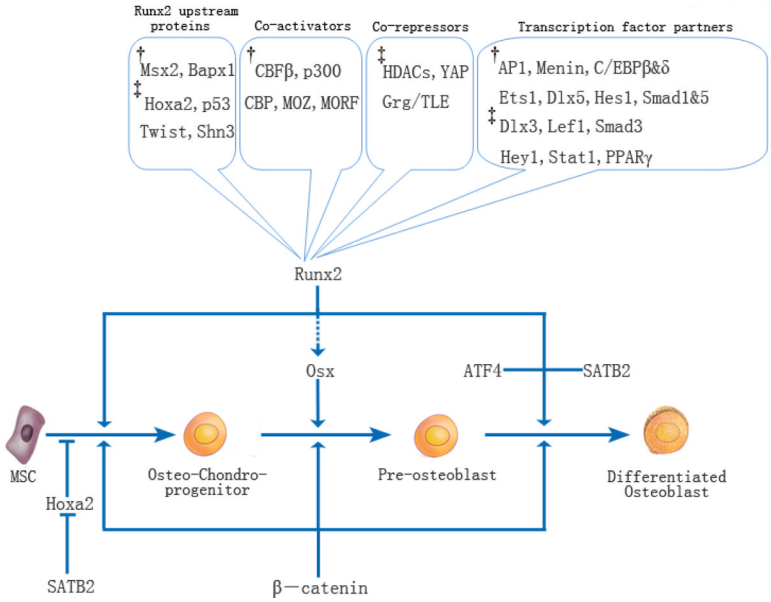

Figure 2.

Regulation of osteoblast differentiation by transcription factors. In osteoblast differentiation, high levels of Runx2 and ß-catenin are necessary to suppress the chondrogenic potential of uncommitted progenitors, such as the proposed osteochondroprogenitor. Osterix is required for the final commitment of progenitors to preosteoblasts. † signs indicate positive effects; ‡ signs indicate inhibitory effects.

Table 1

Genes and their mouse models associated with impaired osteoblast function

Mutated Gene	Defects in Osteoblasts	Role	Reference Mouse Model
Runx2	Devoid of osteoblasts and impaired final differentiation of chondrocytes	Functions as a master switch for inducing osteoblast differentiation	173, 176
	Runx2 interacting transcription factors	Role	Reference Mouse/Cell Model
	AP1	JunB is shown to be essential for osteoblast proliferation and differentiation; Most Fos proteins are also implicated in proliferation and differentiation of osteoblasts	257 – 260
	Smad	Interacts with RUNX2 in vivo and in vitro and enhances the transactivation ability of this factor. The pathogenesis of CCD may be related to impaired Smad signaling	90, 94, 225
	Ets1	Runx2 and Ets1 cooperate in vivo to regulate expression of the Osteopontin (Opn) gene	227
	C/EBPβ and −δ	C/EBP transcription factors support Osteocalcin gene expression and may play an important regulatory role during osteoblast differentiation	228, 229
	Dlx5	Has a critical positive role in osteoblast differentiation and subsequent mineralization.	230,261
	Hes1	Binds to and potentiates the transactivating function of Cbfa1	232
	Menin	Required for bone morphogenetic protein 2- and transforming growth factor beta-regulated osteoblastic differentiation through interaction with Smads and Runx2	233
	Dlx3	Regulating osteoprogenitor cell differentiation and for both positive and negative regulation of gene transcription	231
	Lef1	Lymphocyte enhancer-binding factor 1 (Lef1) inhibits terminal differentiation of osteoblasts.	262
	Msx2	Stimulates the commitment of mesenchymal cells into an osteoblast lineage in association with inhibition of adipogenesis	184 – 186
	PPARγ	Inhibits the Runx2-mediated transcription of osteocalcin in osteoblasts and terminal osteoblast differentiation	238, 263
	Smad3	Decreases Cbfa1 and osteocalcin expression and inhibits osteoblast differentiation.	239
	Hey1	A negative regulator of osteoblast differentiation/ maturation	240
	Stat1	Functions as a cytoplasmic attenuator of Runx2 in the transcriptional program of osteoblast differentiation	241
Osterix	Devoid of osteoblasts.	A critical transcription factor in osteoblast differentiation	7
ATF4	A delay in osteoblast differentiation.	Required for the timely onset and terminal differentiation of osteoblasts	248
SATB2	Defects in osteoblast differentiation.	Acts as a molecular node in a transcriptional network regulating osteoblast differentiation	253
β-catenin	Blocked in differentiation and develops into chondrocytes instead.	Essential for osteoblast differentiation and preventing transdifferentiation of osteoblastic cells into chondrocytes	67, 68, 264

Table 2

Well-established markers of the osteoblast during developmental sequencing.

MSC	Immature Osteoprogenitor	Mature Osteoprogenitor	Preosteoblast	Differentiated osteoblast	Osteocyte
Alkaline Phosphatase (ALP)	−	+	++	+++	−
Phex	−	−	−	+++	+++
Osteocalcin (OCN)	−	−	−	−→+++	−
Osteopontin (OPN)	−/+	−/+	−→+	−→+++	−→+++
Runx2	+	+	++	+++	+++
Osterix	−	−	++	++	?
Collα1	−	++	++	++	−
Bone sialoprotein (BSP)	−↔++	++↔−	−→+++	−→+++	−→+++

The list is not exhaustive, but does show some important categories of molecules in the lineage and their utility to help define transitions in osteoblast differentiation. −, no detectable expression; −/+, +, ++, +++, expression ranging from detectable to very high; −→+++, heterogeneous expression in individual cells.