Transcriptional Control of Skeletogenesis

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Abstract

The skeleton contains three specific cell types: chondrocytes in cartilage and osteoblasts and osteoclasts in bone. Our understanding of the transcriptional mechanisms that lead to cell differentiation along these three lineages has increased considerably in the past ten years. In the case of chondrocytes and osteoblasts advances have been made possible largely through the molecular elucidation of human skeletal dysplasias. This review discusses the key transcription factors that regulate skeletogenesis and highlights their function, mode of action, and regulation by other factors, with a special emphasis on how human genetics has contributed to this knowledge.

INTRODUCTION

Chondrocyte: cell of mesenchymal origin present only in cartilage

Osteoblast: cell of mesenchymal origin present only in bone; responsible for bone formation and controls osteoclast differentiation

The skeleton contains three specific cell types: chondrocytes of various size and shape in cartilage and osteoblasts and osteoclasts in bones. Whereas chondrocytes and osteoblasts are of mesenchymal origin, osteoclasts belong to the monocyte-macrophage cell lineage. In the past two decades extraordinary progress has been made in our understanding of cell differentiation in the skeleton and especially in the identification of transcription factors involved in these events. It is customary to highlight the contribution of animal models, and in particular of mouse genetic studies, in the progress that has been made in the past fifteen years toward the molecular understanding of these processes. This view is justified by the facts, yet it should not obscure the critical contributions of human genetics to the gain of knowledge in this field. This review revisits the main aspects of the transcriptional control of cell differentiation during skeletogenesis by showing how powerful each approach has been and discussing the important combination of these two approaches. Because human genetics has influenced our understanding of the transcriptional control of chondrocyte and osteoblast differentiation significantly, more than it has influenced our understanding of osteoclast differentiation, we focus here on chondrogenesis and osteogenesis.

HUMAN AND MOUSE GENETIC STUDIES OF CHONDROGENESIS

Chondrocytes are the first skeleton-specific cells to appear during embryonic development. Once undifferentiated mesenchymal cells aggregate to form mesenchymal condensations at the location of each future skeletal element, they acquire genetic characteristics of nonhypertrophic chondrocytes (22). The two main features of these resting and proliferating chondrocytes are that they express *Aggrecan* and α_1*(II) collagen*. As skeletogenesis proceeds, proliferating chondrocytes progressively exit the cell cycle, hypertrophy, and become bona fide hypertrophic chondrocytes (25). This latter subset of chondrocytes does not express α_1*(II) collagen* anymore, but instead expresses α_1*(x) collagen*. To date we know much more about the transcriptional control of the early part of chondrogenesis (differentiation of resting and proliferating chondrocytes) than about chondrocyte hypertrophy.

Although many molecular and mouse genetic studies followed, in all fairness this field took off when a human genetic disease marked by severe cartilage abnormalities called campomelic dysplasia was shown to be caused by an inactivating mutation in the gene encoding sex determining region Y (SRY)-box 9 (Sox9) (18, 71) (**Table 1**). This observation put an end to several years of molecular trial and error. Sox9 is a transcription factor that contains a high mobility group (HMG) box, a DNA binding domain that exhibits a high degree of homology with the DNA binding domain of the mammalian testis-determining factor SRY. Following this landmark discovery Sox9 was shown to regulate the expression of *Aggrecan* and α_1*(II) collagen* as well as the expression of α_1*(XI) collagen* and *cartilage-derived retinoic acid-sensitive protein* (*CD-RAP*), two other markers of nonhypertrophic chondrocytes (1, 5, 43, 50, 61, 76). Two experiments further established in vivo the critical importance of Sox9 during chondrogenesis. First, ectopic expression of *Sox9* in vivo is able to transactivate the α_1*(II) collagen* gene in cells not destined to become chondrocytes. Second, *Sox9*$^{-/-}$ embryonic stem (ES) cells are always excluded from the chondrogenic condensations and do not express any of the molecular markers of nonhypertrophic chondrocytes (1, 2). Thus, the conjunction of human genetics, mouse genetics, and molecular studies helped to identify Sox9 as the master gene of chondrogenesis by showing that it controls proliferation and differentiation of nonhypertrophic chondrocytes (**Figure 1**). In addition, Sox9 seems to act as a negative regulator of chondrocyte hypertrophy (3, 27).

Other transcription factors are required or involved in the differentiation of nonhypertrophic chondrocytes; however, until now none have been linked to a particular skeletal

Table 1 Association between human diseases, mouse models, and genes regulating cell differentiation in the skeleton

Gene	Type of factor	Human disease(s)	Mouse model(s)	Process regulated
SOX9	Transcription factor	Campomelic dysplasia	Loss of function Ectopic expression	Chondrocyte differentiation
RUNX2	Transcription factor	Cleidocranial dysplasia	Loss of function Ectopic expression Overexpression	Chondrocyte and osteoblast differentiation
TWIST1	Transcription factor	Sathre-Chotzen syndrome	Loss of function Overexpression	Chondrocyte and osteoblast differentiation
FGFR3	Transmembrane receptor	Achondroplasia Thanatophoric dysplasia	Loss of function Gain of function	Chondrocyte differentiation
MSX2	Transcription factor	Boston-type craniosynostosis Enlarged parietalforamina	Loss of function	Osteoblast differentiation
SATB2	Nuclear matrix protein	Cleft palate	Loss of function	Osteoblast differentiation
RSK2	Protein kinase	Coffin-Lowry syndrome	Loss of function	Osteoblast differentiation and function
NF1	Ras-GTPase activating factor	Neurofibromatosis type I	Loss of function	Osteoblast differentiation and function

dysplasia. Two of these molecules, Sox5 and Sox6, belong to the same family of proteins as Sox9. Both of them can bind to Sox9 and increase Sox9 transactivation function in vitro, although neither Sox5 nor Sox6 has a transactivation domain (44). The importance of these two proteins was verified in vivo: Embryos lacking both Sox5 and Sox6 die at embryonic day 16.5 (E16.5) and display a failure of chondrocyte progenitor cells to differentiate into hypertrophic chondrocytes (44). Besides HMG box–containing transcription factors, hypoxia inducible factor-1 (Hif-1α), a basic helix-loop-helix (bHLH) domain-containing protein, favors chondrocyte survival by regulating the expression of *Vegf*, which encodes a secreted molecule required for vascular invasion of the forming bones (60).

Researchers have also begun to elucidate the transcriptional control of the transition of proliferating chondrocytes into hypertrophic chondrocytes. Here again progress was made largely from the study of genetically engineered mouse models for genes that were initially identified through the molecular elucidation of human skeletal dysplasia. The master gene of osteoblast differentiation, *Runt-related 2* (*Runx2*), whose identification and functional characterization are presented in greater detail below, has a broader role during skeletogenesis, as evidenced by the analysis of the *Runx2*$^{-/-}$ mice. Besides their osteoblast phenotype these mutant mice lack hypertrophic chondrocytes in some but not all skeletal elements (36).

Skeletal dysplasia: genetic disease of the skeleton that affects cell differentiation in all skeletal elements

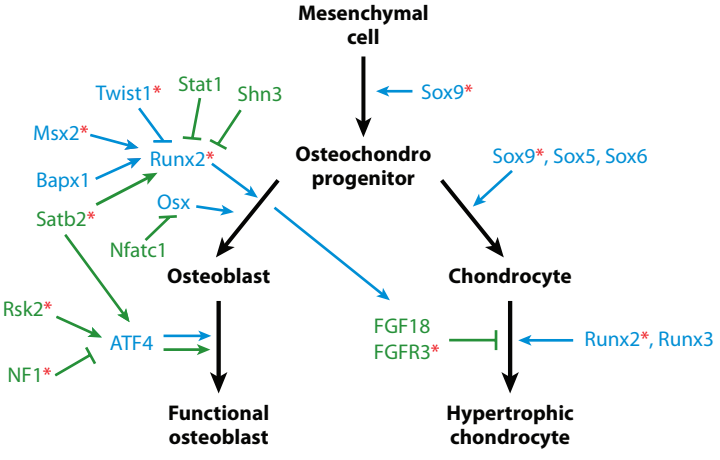

Figure 1

Schematic representation of the transcriptional control of cell differentiation along the chondrocyte and osteoblast lineages. Lines with arrowheads indicate a positive action and lines with bars indicate an inhibition. Regulation at the transcriptional level is shown in blue; regulation at the posttranscriptional level is shown in green. Red asterisks indicate genes whose mutation has been identified as disease-causing in humans.

That skeletal elements containing hypertrophic chondrocytes (such as ulnae) develop later than elements without hypertrophic chondrocytes (like humeri) indicates that this phenotype is not due to a mere delay in cell differentiation. Further analyses showed that *Runx2* is transiently expressed in prehypertrophic chondrocytes, the subpopulation of proliferating chondrocytes that gives rise to the hypertrophic chondrocytes (64). Moreover, constitutive expression of *Runx2* in these cells leads to ectopic chondrocyte hypertrophy in transgenic mice and allows chondrocyte hypertrophy to occur in *Runx2*$^{-/-}$ mice (64). What are the transcription factors that affect chondrocyte hypertrophy in skeletal elements where *Runx2* deletion does not affect chondrocyte hypertrophy? Genetic studies have shown that another member of the Runx2 family of transcription factors, Runx3, is important for chondrocyte hypertrophy. Indeed, mice lacking both Runx2 and Runx3 do not have recognizable hypertrophic chondrocytes or α_1*(X) collagen*–expressing cells anywhere in the skeleton (79). Therefore, two members of the Runx family of transcription factors, Runx2 and Runx3, are positive regulators of chondrocyte hypertrophy (**Figure 1**).

The study of another transcription factor, Twist-1, added some complexity to this picture. The function of Twist-1during skeletogenesis was also revealed via the molecular elucidation of a human skeletal dysplasia, Saethre-Chotzen syndrome (16, 26) (**Table 1**). Twist-1 is a nuclear protein containing at least two known functional structures: a bHLH domain and the Twist-box, a domain located at the C terminus that mediates the physical interaction of Twist-1 with Runx2 and inhibits the ability of this transcription factor to bind to DNA (4). Both Twist-1 and Runx2 are expressed in undifferentiated cells that form the perichondrium, a multilayer sheath of cells that surrounds the growth plate cartilage and inhibits chondrocyte hypertrophy. Analyses of loss- and gain-of-function mouse models showed that Twist-1 favors chondrocyte hypertrophy through its perichondrial expression and that this function requires Runx2 (4, 24). Further studies showed that Runx2 regulates positively the expression of *fibroblast growth factor 18* (*Fgf18*) in the perichondrium (24). FGF18, a secreted molecule, then activates FGFR3 signaling in chondrocytes, thereby inhibiting chondrocyte hypertrophy (46, 52) (**Figure 1**). Human geneticists identified the function of FGFR3 in chondrocytes in the 1990s because activating mutations in this gene cause achondroplasia and thanatophoric dysplasia (49, 55, 62, 66) (**Table 1**). Therefore, Runx2 has two opposite functions during chondrocyte hypertrophy. Initially, through its transient expression in prehypertrophic chondrocytes, Runx2 induces chondrocyte hypertrophy and sets up the scene for the next events of skeletogenesis, i.e., vascular invasion and osteoblast differentiation. Subsequently, through its constitutive expression in the cells of the perichondrium, Runx2 inhibits chondrocyte proliferation and hypertrophy, possibly to avoid premature bone formation.

In summary, it is remarkable that the two main transcriptional architects of chondrogenesis, Sox9 and Runx2, were identified through the joint efforts of human geneticists, molecular biologists, and mouse geneticists. As presented below, the same is true for the transcriptional control of osteoblast differentiation.

TRANSCRIPTIONAL CONTROL OF OSTEOBLAST DIFFERENTIATION

Multiple nuclear proteins contribute to the regulation of osteoblast differentiation and function. Some of them act throughout the skeleton, others act only in a subset of skeletal elements, and members of a third category modulate the activity of classical transcription factors. Remarkably, many molecular determinants of osteoblast differentiation and function were identified through human genetic studies as well as through mouse genetics and molecular studies.

Runx2, the Master Gene of Bone Formation

Runx2, a member of the Runt domain family of transcription factors, was shown to be the earliest and most powerful molecular determinant of osteoblast differentiation simultaneously by molecular biologists, mouse geneticists, and human geneticists. A classical cell-specific promoter-based search for osteoblast-specific transcription factors identified Runx2 as the factor binding to an osteoblast-specific *cis*-acting element in the promoter of the genes coding for osteocalcin, an osteoblast-specific hormone that regulates energy metabolism (11, 12, 42). Runx2 has all the molecular hallmarks of an osteoblast differentiation factor. In particular, Runx2 is expressed in cells prefiguring the skeleton as early as E10.5 (9). At that stage, these cells still have the capacity to differentiate into osteoblasts or chondrocytes and therefore are termed osteochondro progenitors (**Figure 1**). Subsequently, while its expression in differentiating chondrocytes decreases and eventually vanishes (at E16.5) Runx2 remains expressed at high levels in cells of the osteoblast lineage and of the perichondrium (4, 12, 24). Runx2 regulates many but not all genes that determine the osteoblast phenotype. Remarkably, forced expression of Runx2 in nonosteoblast cells is sufficient to induce the expression of many osteoblast-specific genes such as *Osteocalcin* (12). Consistent with its pattern of expression and its function in vitro, inactivation of both *Runx2* alleles in mice results in a mutant mouse deprived of osteoblasts throughout the skeleton (40, 53). Moreover, haploinsufficiency at the *Runx2* locus results in mice with hypoplastic clavicles and delayed closure of the fontanelles (47, 53). These latter abnormalities are similar to what is seen in a human skeletal dysplasia called cleidocranial dysplasia (CCD) (**Table 1**). Indeed, *Runx2* maps in the middle of the CCD locus and molecular studies demonstrated that most forms of CCD are due to inactivating mutations in *Runx2* (41, 47, 80). Therefore, this overwhelming molecular and genetic evidence accounts for the widely accepted view that Runx2 is the master gene of osteoblast differentiation. However, as presented above, the functions of Runx2 extend to other aspects of skeletogenesis.

Regulators of Runx2: A Tale of Human and Mouse Genetics

Given the critical functions that Runx2 exerts during skeletogenesis, it is not surprising that its activity, if not its expression, is tightly regulated. Many factors have been shown to affect the ability of Runx2 to bind to DNA and/or to regulate its transactivation function; the order in which they are presented here does not reflect a ranking of their biological importance but rather serves to illustrate how human genetics has been an engine in deciphering the transcriptional control of cell differentiation during skeletogenesis.

Molecularly Runx2 was identified on the basis of its ability to regulate the expression of *Osteocalcin*, an osteoblast-derived hormone expressed only in fully differentiated osteoblasts (12, 42). However, during mouse development *Osteocalcin* expression does not appear before E15.5, i.e., four to five days after Runx2 expression can be detected. One possible explanation for this paradox could be that during these four to five days Runx2 function is transiently inhibited by another nuclear protein. One way to identify such a protein is to use human genetics observations to single out candidate genes. Haploinsuffiency at the *Runx2* locus leads to delayed ossification of the skull bones (47, 53). Conceivably, inactivation of a gene whose function is to inhibit Runx2 function should thus lead to an increase in bone formation in the skull, a condition called craniosynostosis (72). Among the genes whose inactivation causes such a phenotype only one encodes for a nuclear protein, Twist-1. Indeed, haploinsufficiency at the *Twist-1* locus causes Saethre-Chotzen syndrome, a form of craniosynostosis (16, 26) (**Table 1**). Human genetics therefore identified Twist-1 as a candidate gene and opened the way for mouse genetics and molecular studies. These analyses verified that Twist-1

indeed delays osteogenesis via an inhibition of Runx2 activity (4). The researchers showed that (*a*) during early development *Twist-1* is transiently coexpressed with *Runx2* in cells destined to become osteoblasts and its expression disappears in these cells precisely when osteoblast differentiation is initiated; (*b*) the Twist box binds to the Runx2 runt domain (DNA binding domain) and inhibits Runx2 binding to DNA; (*c*) removing one allele of *Twist-1* from $Runx2^{+/-}$ mice is sufficient to correct the skull phenotype of each single heterozygous mutant mouse; and (*d*) an N-ethyl-N-nitrosourea (ENU) mutant in which the Twist box is disrupted displays premature osteoblast differentiation and acceleration of chondrocyte hypertrophy. That Twist-1 is an inhibitor of Runx2 would likely have been shown sooner or later but human genetics helped to quickly identify the best candidate that could inhibit Runx2 functions early during development (**Figure 1**).

Other regulators of Runx2 function have been identified. The role of the homeobox-containing protein muscle segment homeobox (msh) homolog (MSX2) during skeletal development was demonstrated when gain- and loss-of-function mutations were identified in human patients with either Boston-type craniosynostosis or enlarged parietal foramina, respectively (28, 73) (**Table 1**). Accordingly, *Msx2*-deficient mice display defective ossification of the skull and bones that develop by endochondral ossification (58). Because the expression of *Osteocalcin* and *Runx2* is strongly reduced in *Msx2*-deficient mice, Msx2 was proposed to act upstream of Runx2 in a transcriptional cascade that regulates osteoblast differentiation (**Figure 1**). A similar observation was made for mice lacking the homeodomain-containing transcription factor bagpipe homeobox gene 1 homolog (Bapx1) (68). These mice die at birth owing to severe dysplasia of the axial skeleton, whereas the appendicular skeleton is virtually unaffected. *Runx2* expression in *Bapx1*-deficient mice is strongly reduced in osteochondrogenic precursor cells of the prospective vertebral column, indicating that Bapx1 is required for *Runx2* expression specifically in these skeletal elements (**Figure 1**). Other nuclear proteins inhibit Runx2 function during osteoblast differentiation by interacting physically with the Runx2 DNA-binding domain. One of these proteins is signal transducer and activator of transcription 1 (Stat1), a transcription factor regulated by extracellular signaling molecules such as interferons. *Stat1*-deficient mice are viable but develop a high-bone-mass phenotype explained by enhanced bone formation (37). The increase of osteoblast differentiation and function in these mice is molecularly explained by the lack of a Stat1-mediated inhibition of the transcriptional activity of Runx2 (**Figure 1**). Interestingly, the physical interaction of both proteins is independent of Stat1 activation by phosphorylation. Stat1 has been proposed to act by inhibiting the translocation of Runx2 into the nucleus because overexpression of Stat1 in osteoblasts leads to cytosolic retention of Runx2 whereas nuclear translocation of Runx2 is much more prominent in *Stat1*-deficient osteoblasts (37). Schnurri 3 (Shn3) is another protein that interacts with Runx2 and acts by decreasing the availability of Runx2 in the nucleus (**Figure 1**). Shn3 is a zinc finger adapter protein originally thought to be involved in the VDJ recombination of immunoglobulin genes (74). A *Shn3*-deficient mouse model unexpectedly revealed a major function of this protein in bone formation. These mice display a severe adult-onset osteosclerotic phenotype owing to a cell-autonomous increase of bone matrix deposition (31). Interestingly, whereas several Runx2 target genes are expressed at higher rates in *Shn3*-deficient osteoblasts, *Runx2* expression is not affected by the absence of Shn3. However, Runx2 protein levels increase strikingly in *Shn3*-deficient osteoblasts. This latter finding is molecularly explained by the function of Shn3 as an adapter molecule linking Runx2 to the E3 ubiquitin ligase WW domain–containing protein 1 (WWP1) (31). The Shn3-mediated recruitment of WWP1 in turn leads to an enhanced proteasomal degradation of Runx2. This mechanism is best underscored by the finding that RNAi-mediated

downregulation of WWP1 in osteoblasts leads to increased Runx2 protein levels and enhanced extracellular matrix mineralization, thereby virtually mimicking the defects observed in the absence of Shn3 (31). Altogether, these data identify Shn3 as a key regulator of Runx2 actions in vivo (**Figure 1**). Moreover, given the postnatal onset of the bone phenotype of the *Shn3*-deficient mice, compounds blocking the interaction of Runx2, Shn3, and WWP1 may serve as specific therapeutic agents for the treatment of bone loss diseases such as osteoporosis.

In addition to negative regulators of *Runx2* function, interacting factors that enhance Runx2 activity also exist. One of them is the nuclear matrix protein special AT-rich sequence-binding protein 2 (SATB2). The importance of this protein in skeletogenesis was first discovered in human patients with cleft palate who carry a heterozygous chromosomal translocation that inactivates the *SATB2* gene (17) (**Table 1**). The generation of a *Satb2*-deficient mouse model confirmed the importance of this gene in craniofacial development, skeletal patterning, and osteoblast differentiation (8). The latter function was in part attributed to an increased expression of *homeo box A2* (*Hoxa2*), a negative regulator of prechondrogenesis and bone formation (32), whose expression is repressed by the binding of Satb2 to an enhancer element present in the *Hoxa2* promoter (8). In addition to this type of action, there is also a Hoxa2-independent influence of Satb2 on the transcription of *Bone Sialoprotein* and *Osteocalcin*. Whereas in the case of *Bone Sialoprotein* Satb2 directly binds to an osteoblast-specific promoter element, the activation of *Osteocalcin* expression by Satb2 requires a physical interaction with Runx2 (8) (**Figure 1**). This requirement was demonstrated by cotransfection assays using an osteoblast-specific *Osteocalcin* promoter fragment and by coimmunoprecipitation experiments. Moreover, the synergistic action of Satb2 and Runx2 in osteoblasts was genetically confirmed by the generation of compound heterozygous mice lacking one allele of each gene (8). These results identified Satb2 as an important regulator of osteoblast differentiation in both mice and humans. Moreover, the finding that Satb2 also interacts with activating transcription factor-4 (ATF4), another transcription factor involved in the regulation of osteoblast differentiation and function that is discussed below (8), illustrates that the transcriptional network regulating bone formation is much more complex than previously anticipated.

Osterix, a Runx2-Dependent Osteoblast-Specific Transcription Factor Required for Bone Formation

Besides Runx2, there is at least one more transcription factor, termed Osterix (Osx), whose activity is absolutely required for osteoblast differentiation in mice (**Figure 1**). Osx is a zinc finger–containing transcription factor that is specifically expressed in osteoblasts of all skeletal elements (48). Inactivation of Osx in mice results in perinatal lethality owing to a complete absence of bone formation (48). Unlike *Runx2*-deficient mice whose skeleton is entirely nonmineralized, the *Osx*-deficient mice lack a mineralized matrix only in bones formed by intramembranous ossification. The *Osx*-deficient bones formed by endochondral ossification contain some mineralized matrix, although it resembles calcified cartilage, not mineralized bone matrix (48). This finding shows that Osx, unlike Runx2, is not required for chondrocyte hypertrophy, thereby demonstrating that Osx specifically induces osteoblast differentiation and bone formation in vivo (**Figure 1**). Comparative expression analyses by in situ hybridization further revealed that *Osx* is not expressed in *Runx2*-deficient embryos, whereas *Runx2* is normally expressed in *Osx*-deficient embryos (48). These results demonstrated that Osx acts downstream of Runx2 in the transcriptional cascade of osteoblast differentiation, and that Osx expression could be directly regulated by the binding of Runx2 to a responsive element in the promoter of the *Osx* gene (51). Unlike for *Runx2*, no mutations of the human *Osx* gene have been identified that

would be associated with decreased bone formation. Moreover, in contrast to the steadily increasing knowledge about the function of Runx2 and its regulation by other molecules, the molecular mechanisms underlying the action of Osx in osteoblasts are less well understood. Nevertheless, one recent publication provides evidence for a contribution of Osx to the negative effects of nuclear factor of activated T cells (NFAT) inhibitors on bone mass (39). NFAT inhibitors, such as FK506 or cyclosporin A, are commonly used as immunosuppressants, for example after organ transplantation (45). However, this treatment is often accompanied by the development of osteopenia in the receptive patients (54). Likewise, treatment of mice with FK506 leads to decreased bone mass owing to impaired bone formation, and the same phenotype is observed in mice lacking the transcription factor Nfatc1 (39). The deduced role of Nfatc1 as a physiological activator of osteoblast differentiation and function can be molecularly explained by an interaction with Osx. In fact, both proteins synergistically stimulate the activity of an osteoblast-specific *α1(I)-Collagen* promoter fragment via the formation of an Nfatc1/Osx DNA-binding complex (39) (**Figure 1**). The complexity of the transcriptional control of osteoblast differentiation will likely increase further when more Osx-interacting molecules are identified.

AP1 Regulation of Osteoblast Differentiation and Function

Activator protein 1 (AP1) proteins are heterodimeric transcription factors composed of members of the Jun and Fos family of basic leucine zipper proteins (33). These proteins include the Jun proteins c-Jun, JunB, and JunD, as well as the Fos proteins c-Fos, Fra1, Fra2, and Fosb. Although AP1 transcription factors fulfill various functions in different cell types, it is striking that some family members play specific roles in bone remodeling, as demonstrated by several loss- or gain-of-function studies in mice (30, 70). For instance, the deletion of *c-Fos* from the mouse genome results in severe osteopetrosis owing to an arrest of osteoclast differentiation, whereas overexpression of *c-Fos* in transgenic mice results in osteosarcoma development (20, 21). Moreover, transgenic mice overexpressing either *fos-related antigen 1* (*Fra1*) or Δ*fosB*, a splice variant of *FosB*, display a severe osteosclerotic phenotype caused by increased osteoblast differentiation and function (29, 57). Likewise, mice lacking Fra1 in extraplacental tissues display an osteopenia associated with reduced bone formation, indicating a physiological role of Fra1 in osteoblasts (13). When the same approach was used to inactivate JunB in extraplacental tissues, thereby circumventing the embryonic lethality caused by a complete genomic deletion of *JunB*, the resulting mice developed a state of low bone turnover due not only to cell-autonomous defects of osteoblasts but also to abnormal osteoclast differentiation (35). Taken together, these data provide evidence for a role of AP1 transcription factors in the regulation of bone formation, although their connection to the other transcriptional regulators described above or below still needs further investigation. For instance, it is known from other cell types that Jun proteins can also interact with ATF family members, thus raising the possibility that heterodimerization with ATF4 may be one mechanism by which these proteins can regulate osteoblast-specific gene expression (6). Interestingly, David and coworkers (7) recently demonstrated that the osteosarcoma development of *c-Fos* transgenic mice is dramatically decreased in a *p90 ribosomal S6 protein kinase 2* (*Rsk2*)-deficient genetic background. This observation is molecularly explained by the lack of c-Fos phosphorylation by Rsk2, leading to increased proteosomal degradation. Thus, Rsk2 is apparently not only involved in the physiological regulation of bone formation via phosphorylation of ATF4, but also may have an influence on the development of osteosarcomas via phosphorylation of c-Fos.

Another mechanism by which AP1 family members might be involved in the regulation of bone formation was identified by the analysis of mouse models with impaired circadian

regulation; this mechanism links AP1 family members to the regulation of bone mass by leptin (10, 19, 34). Mutant mice that lack components of the molecular clock, namely the *period* (*Per*) or *cryptochrome* (*Cry*) genes, display a high-bone-mass phenotype caused by increased bone formation and respond to intracerebroventricular infusion of leptin with an increase instead of a decrease in bone mass (19). These results suggest that the components of the molecular clock are involved in the regulation of bone formation via the sympathetic nervous system (SNS), a known mediator of leptin's actions on bone mass (34, 65). Interestingly, virtually all genes encoding members of the AP1 transcription factor family are expressed at higher levels in osteoblasts derived from mice lacking either the *Per* genes or the β2-adrenergic receptor Adrβ2, the receptor targeted by the SNS in osteoblasts, compared with wild type (19, 65). This increase is especially pronounced in the case of the *c-Fos* gene, whose expression can also be induced by the addition of isoproterenol, a sympathomimetic, in wild-type osteoblasts (19). In turn, c-Fos leads to a direct activation of *c-Myc* transcription, thereby indirectly increasing the intracellular levels of cyclin D1 and promoting osteoblast proliferation (19). These data demonstrate that the expression of AP1 components is activated via sympathetic signaling, and the activity of clock gene products counteracts this induction.

ATF4, a Regulator of Osteoblast Function Implicated in Two Human Diseases

This review ends with a discussion of the transcription factor ATF4, which seems to play the most important role in assuring that osteoblasts fulfill their function. In this case the biological importance of this factor was revealed by human genetics as much as by any other type of approach. Studies that led to its identification started with the following question: How can inactivation of a kinase decrease bone mass? *RSK2*, which encodes a kinase, is the gene mutated in Coffin-Lowry syndrome, an X-linked mental retardation condition associated with skeletal abnormalities (69) (**Table 1**). Likewise, *Rsk2*-deficient mice display decreased bone mass owing to impaired bone formation (78). In vitro kinase assays demonstrated that ATF4 is more strongly phosphorylated by Rsk2 than any other proposed substrate, and that this phosphorylation is undetectable in osteoblasts derived from *Rsk2*-deficient mice. The subsequent analysis of an *ATF4*-deficient mouse model revealed that this transcription factor plays several crucial roles in osteoblast differentiation and function (**Figure 1**); *ATF4*-deficient mice display a delayed skeletal development and thereafter develop a severe low-bone-mass phenotype caused by decreased bone formation (78).

Molecularly, ATF4 was identified as the factor binding to an osteoblast-specific element in the *Osteocalcin* promoter, thereby directly activating the transcription of the *Osteocalcin* gene (11, 59, 78). Moreover, ATF4 is required for proper synthesis of type I collagen (which seems to be the main mechanism whereby ATF4 regulates bone formation), although this function is not mediated by a transcriptional regulation of *type I collagen* expression (78). In fact, because type I collagen synthesis is specifically reduced in primary osteoblast cultures lacking ATF4, but this defect can be rescued by adding nonessential amino acids to the culture, ATF4 appears to be required for efficient amino acid import into osteoblasts, as described for other cell types (23). Reduced type I collagen synthesis was subsequently observed in mice lacking Rsk2, providing evidence that the diminished ATF4 phosphorylation in the absence of Rsk2 may contribute to the skeletal defects associated with Coffin-Lowry syndrome (78).

In addition to the role of ATF4 in bone formation, ATF4 regulates osteoclast differentiation and ultimately bone resorption through its expression in osteoblasts (14). This function is molecularly explained by the binding of ATF4 to the promoter of the *receptor activator of nuclear factor-KB ligand* (*Rankl*) gene, which encodes a factor secreted by osteoblasts that promotes osteoclast

differentiation (67). Accordingly, *ATF4*-deficient mice have decreased osteoclast numbers owing to reduced *Rankl* expression. Most importantly, this function of ATF4 is involved in the control of bone resorption by the SNS (14). In fact, treatment of normal osteoblasts with isoprotenerol, a surrogate of sympathetic signaling, enhances osteoclastogenesis of cocultured bone marrow macrophages through an induction of osteoblastic *Rankl* expression (14). As expected, this effect is blunted when the osteoblasts are derived from mice lacking the β2-adrenergic receptor Adrβ2. However, the effect of isoprotenerol is also blunted by an inhibitor of protein kinase A, or by using osteoblasts derived from *ATF4*-deficient mice (14). Taken together, these results demonstrate that ATF4 is an important mediator of extracellular signals, such as β-adrenergic stimulation, in osteoblasts.

Thus, it is not surprising that the function of ATF4 is regulated mostly posttranslationally. For example, as mentioned above, ATF4 also interacts with other proteins, such as the nuclear matrix protein Satb2 (8). As described above, the proximal *Osteocalcin* promoter contains two osteoblast-specific elements that serve as binding sites for ATF4 and Runx2, respectively (11, 12, 59, 78). Owing to the proximity of both elements a physical interaction of the two proteins occurs that is stabilized by Satb2, which acts as a scaffold to enhance the synergistic activity of Runx2 and ATF4 in the regulation of *Osteocalcin* expression (8, 75).

Other aspects of ATF4 biology are also regulated posttranslationally. In fact, even the osteoblast-specificity of ATF4 function is not determined by osteoblast-specific *ATF4* expression, but by a selective accumulation of the ATF4 protein in osteoblasts, which is explained by the lack of proteosomal degradation (77). This concept is best demonstrated by the finding that the treatment of nonosteoblastic cell types with the proteasome inhibitor MG115 leads to an accumulation of the ATF4 protein, thereby resulting in ectopic *Osteocalcin* expression (77). These data provide the first evidence that the cell-specific function of a transcriptional activator can be achieved by a posttranslational mechanism. This finding is of general importance for our understanding of the transcriptional networks that control cellular differentiation and function because it may relate to other factors that regulate these processes.

As mentioned above and illustrated in the case of Coffin-Lowry syndrome, ATF4 biology illustrates how the molecular understanding of a disease-causing gene can translate to therapeutic interventions. This concept was further established by the finding of a link between an increased Rsk2-dependent phosphorylation of ATF4 and the development of the skeletal abnormalities in human patients suffering from neurofibromatosis (56, 63) (**Table 1**). This disease, primarily known for tumor development in the nervous system, is caused by inactivating mutations of the *neurofibromatosis 1* (*NF1*) gene, which encodes a Ras-GTPase activating protein (38). The generation of a mouse model lacking *Nf1* specifically in osteoblasts ($Nf1_{ob-/-}$ mice) led to the demonstration that this gene plays a major physiological role in bone remodeling (**Figure 1**). The $Nf1_{ob-/-}$ mice display a high bone mass phenotype caused by increased bone turnover accompanied by an enrichment of nonmineralized osteoid (15). Further analysis of this phenotype revealed that the lack of NF1 induces an increased production of type I collagen, which is molecularly explained by a Rsk2-dependent activation of ATF4. Accordingly, transgenic mice overexpressing *ATF4* in osteoblasts display a phenotype similar to the $Nf1_{ob-/-}$ mice, and the increased type I collagen production and osteoid thickness in the $Nf1_{ob-/-}$ mice are significantly reduced by haploinsufficiency of ATF4 (15).

These molecular findings may also have therapeutic implications. Given the previously discussed function of ATF4 in amino acid import, it appeared reasonable to analyze whether the skeletal defects of the $Nf1_{ob-/-}$ mice could be affected by dietary manipulation. Indeed, the increased bone formation and osteoid thickness of $Nf1_{ob-/-}$ mice can be normalized by a low-protein diet, and the same is true for the phenotype of the transgenic mice that overexpress

ATF4 in osteoblasts (15). Conversely, the defects of osteoblast differentiation and bone formation observed in both the *ATF4*- and the *Rsk2*-deficient mice can be corrected by a high-protein diet (15). These data not only underscore the importance of ATF4 in osteoblast biology, but also demonstrate how the knowledge about its specific functions in osteoblasts can be useful for the treatment of skeletal diseases.

In summary, if we look at a cascade of cell differentiation in the skeleton we now have a fairly detailed picture of the identity and mechanisms of action of many transcription factors involved in these processes. What is remarkable, and was not necessarily anticipated at the beginning of this journey, is how many of these transcription factors are either mutated or their activities affected in human skeletal dysplasia.

SUMMARY POINTS

1. Chondrogenic differentiation of condensed mesenchymal cells, orchestrated by sex determining region Y (SRY)-related high mobility group-box gene (Sox9) is the initial event in skeletogenesis.
2. Sox9, Sox5, and Sox6 determine further differentiation in the chondrocyte lineage.
3. Runt-related 2 (Runx2) is the master gene of osteoblast differentiation.
4. Runx2 also controls chondrocyte maturation via two distinct mechanisms.
5. ATF4 is the major determinant of osteoblast function.

FUTURE ISSUES

1. How is Sox9 regulated at the transcriptional and posttranscriptional level?
2. How many more proteins interact with Runx2 in osteoblasts and chondrocytes?
3. How is Osterix (Osx) regulated at the transcriptional and posttranscriptional level?
4. How are the functions of activator protein 1 (AP1) factors connected with Runx2, Osx, and activating transcription factor-4 (ATF4) activities?
5. Do we know all the functions of ATF4?

DISCLOSURE STATEMENT

The author is not aware of any biases that might be perceived as affecting the objectivity of this review.

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