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PERSPECTIVES

Update on the Transcriptional Control of Osteoblast Differentiation

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Abstract

2007 marks the tenth anniversary of the identification of Runx2 as a master regulator of osteogenesis. These few papers defined precisely when the field of the transcriptional control of osteoblast differentiation really took off. In this ten-year span, our understanding of the transcriptional control of osteoblast differentiation has made unforeseen progress that will be summarized here. This progress was driven, in part, by a combination of molecular biology and mouse genetic approaches. This was predictable. What was less predictable, yet turned out to exert a profound and long-lasting influence on the field, has been the role played by human genetics as illustrated by the large number of key players in the cell differentiation process that are either mutated or have their activity affected in genetically inherited skeletal diseases. Clearly the role of clinical information in identifying some of these factors has been more pivotal that one would have anticipated in 1997. In turn, elucidating the molecular mechanism of action of osteoblast-specific transcription factors has resulted in novel and simple therapeutic strategies proposed for at least two skeletal dysplasias. *BoneKEy*. 2007 Jun;4(6):164-170.
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Runx2 as a General Regulator of Skeletogenesis

A decade has now passed since the identification of Runx2, the first osteoblast-specific transcription factor to be discovered (1-5). Runx2 also turned out to be the earliest, most specific and, for now, most important determinant of osteoblast differentiation. Runx2 is a member of the Runt family of transcription factors, but unlike the other members of this family does not need to heterodimerize with CBFB to regulate gene expression, as was shown in cell-based assays and, as will be mentioned later, genetically (6-8). *Runx2* expression is essentially limited to cells of mesenchymal origin prefiguring the future skeleton. In 10.5 dpc mouse embryos, it marks a cell population that can become either osteoblasts or chondrocytes, which by 1997 had already been dubbed osteochondroprogenitor cells. (3). Later on, *Runx2* is constitutively expressed in osteoblasts regardless of their stage of differentiation (3). *Runx2* is not only expressed in the right cells at the right time, but it also regulates the expression of most genes expressed in osteoblasts, including osteoblast-specific genes such as the osteocalcin gene that served as a tool to identify it as a regulator of osteoblast gene expression (9). Runx2 forced expression in other cell types of mesenchymal origin leads to osteoblast differentiation, and its inactivation in mice gives rise to animals without any osteoblasts anywhere in the skeleton (1;5). Together, these latter two experiments established that Runx2 is both necessary and sufficient for osteoblast differentiation. Haploinsufficiency at the Runx2 locus gives rise to a mouse lacking most of its clavicles and part of the skull, a phenotype that is a phenocopy of a human disease called cleidocranial dysplasia (1;5). As expected given these molecular and mouse genetic observations, haploinsufficiency at the Runx2 locus in humans causes cleidocranial dysplasia (2;4). Remarkably, although its complete deletion has some effect on bone development, haploinsufficiency at the

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CBFB locus does not lead to a cleidocranial dysplasia phenotype, further differentiating the two molecules, at least *in vivo* (7). We know very little about the regulation of *Runx2* expression, yet the most definitive studies performed so far have shown that Wnt signaling acts early to regulate osteoblast differentiation by regulating Runx2 expression (10-13).

Runx2 expression during skeletogenesis precedes osteoblast differentiation by at least 4 to 5 days. This long delay is explained by the transient co-expression in Runx2-expressing cells of nuclear proteins acting as inhibitors of Runx2 functions, namely Twist-1 in the craniofacial skeleton, and Twist-2 in the appendicular skeleton (14). Before being verified through molecular means and mouse genetics analyses, the notion that the Twist proteins are the initial gatekeepers of skeletogenesis was suggested by clinical observations (15;16). Indeed, the Saethre-Chotzen syndrome, a disease characterized by craniosynostosis, i.e., excessive osteoblast proliferation in the skull, is caused by haploinsufficiency at the *Twist-1* locus, in other words, by an increase in Runx2 activity. The Twist protein belongs to an ever-growing number of molecules that can interact with and offset the function of Runx2 (17). For some of these Runx2-interacting proteins, *in vivo* evidence has verified that they affect skeletal development or bone mass post-natally. Among them one should cite, because of its unique mode of action, Schnurri 3, a zinc finger protein that controls Runx2 protein levels by promoting its degradation through the recruitment of the E3 ubiquitin ligase WWP1 (18). This mode of action holds great promise for the development of Schnurri inhibitors for the treatment of osteoporosis.

Runx2 function during skeletogenesis is limited neither to osteoblast differentiation nor to favoring cell differentiation. First, through its transient expression in prehypertrophic chondrocytes, Runx2 is required, alone in some skeletal elements or with another Runx protein (Runx3) in others, for the differentiation of hypertrophic chondrocytes (19-21). Second, in addition to its well-established pro-differentiation ability, Runx2 is also an inhibitor of chondrocyte and osteoblast differentiation. Specifically, through its expression in cells of the bone collar and the perichondrium, Runx2 regulates expression of a secreted molecule, FGF18, which in turn inhibits osteoblast and chondrocyte differentiation (22-24). Altogether, this broad spectrum of functions gives to Runx2 all the properties of a general transcriptional architect of skeletogenesis with the ability, by acting at different time points during embryogenesis, to insure that all phases of skeletogenesis – chondrocyte differentiation, chondrocyte hypertrophy and osteoblast differentiation – occur in an ordered manner.

Transcriptional Factors Acting Downstream of Runx2

Given its early expression during skeletogenesis, one of the challenges in the field has been to identify transcription factors acting downstream of *Runx2*. *Osterix* is probably the gene that is the most immediately downstream of Runx2 in the pathway leading to osteoblast differentiation. *Osterix* encodes a zinc-finger containing protein that is a member of the Sp family. It was originally identified in a screen for bone morphogenic protein (Bmp)-regulated genes in a cell line. Whether *Osterix* is a Bmp target gene remains unknown, but genetic evidence demonstrated that it is a major determinant of osteoblast differentiation (25). *Osterix*-deficient mice, like *Runx2*-deficient mice, do not have any osteoblasts, thus establishing in the most definitive manner the importance of Osterix during this process. *Osterix* is not expressed in *Runx2*-deficient mice, whereas *Runx2* is expressed in *Osterix*-deficient mice, indicating that *Osterix* acts downstream of *Runx2*. Unlike the case of *Runx2,* haploinsufficiency for *Osterix* does not have an overt effect on osteoblast differentiation. No human disease has yet been shown to be caused by loss-of-function mutations in *Osterix*, and the list of *Osterix* target genes is, for now, relatively limited. This is likely to change in the future.

Although *in vivo* evidence indicates that Runx2 regulates bone formation by differentiated osteoblasts (26), it has been

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suggested from the inception of the field that another osteoblast-specific transcription factor may exist. This presumption was based on systematic study of the osteocalcin promoter, which showed the existence of two *cis*-acting elements, OSE2, to which Runx2 binds, and OSE1 (9). Identification of the OSE1-binding protein relied on molecular effort, combined with mouse and human genetic investigations. Those studies led to the identification of the leucine-zipper containing protein ATF4, which is highly enriched in osteoblasts, as the factor binding to OSE1 (27). An increasing amount of information available about ATF4 suggests that its role in osteoblast functions may approach in importance the role of Runx2 during osteoblast differentiation. At the molecular level, ATF4 regulates bone formation by regulating amino acid import (27). This function requires that ATF4 is phosphorylated by a particular kinase, Rsk2, which is inactivated in a rare human disease, the Coffin-Lowry syndrome. In contrast, Rsk2 and thereby ATF4 activity is increased in osteoblasts in another disease, neurofibromatosis type 1 (28). The regulation of amino acid import by ATF4 was subsequently used to design a diet-based treatment for animal models of Coffin-Lowry syndrome and of neurofibromatosis type 1, thus illustrating how the knowledge of the molecular mechanism of action of a transcription factor can have therapeutic implications (28).

ATF4 does not only regulate bone formation but also expression in osteoblasts of *TNF11*, a gene encoding the key osteoclast differentiation factor, RANK ligand (29). This function of ATF4 occurs following its phosphorylation by a different kinase, PKA, and serves to mediate the leptin-dependent sympathetic regulation of osteoclast differentiation. As expected, this latter function of ATF4 is not modulated by any diet manipulation.

AP1 Regulation of Osteoblast Differentiation and Function

Activator protein 1 (AP1) is a heterodimeric transcription factor composed of members of the Jun and Fos family of basic leucine zipper proteins (30). These include the Jun proteins c-Jun, JunB and JunD, as well as the Fos proteins c-Fos, Fra1, Fra2 and Fosb, respectively. That some of the family members play important roles in bone remodeling is demonstrated by several loss- or gain-of function studies in mice (31). For instance, the deletion of *c-Fos* from the mouse genome results in severe osteopetrosis due to an arrest of osteoclast differentiation, while the transgenic over-expression of *c-Fos* results in osteosarcoma development (32;33). Likewise, mice over-expressing either *Fra1*, or *ΔfosB*, a splice variant of FosB, display a severe osteosclerotic phenotype (34;35), while mice lacking Fra1 in extraplacental tissues display osteopenia associated with reduced bone formation (36). Inactivation of JunB in extraplacental tissues leads to low bone mass (37).

Taken together, these data provide evidence for a crucial role of AP1 transcription factors in the regulation of bone formation. Although their connection to the other transcriptional regulators described here is still not clear, it is known 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 (38). It has also been shown that the development of osteosarcoma in *c-Fos* transgenic mice is weakened by *Rsk2*-deficiency (39). This is explained by the lack of c-Fos phosphorylation by Rsk2, thereby leading to increased proteosomal degradation, Thus, Rsk2 is apparently not only involved in the physiological regulation of bone formation via phosphorylation of ATF4, but may also have an influence on the development of ostosarcomas via phosphorylation of c-Fos.

Another mechanism by which AP1-family members might be involved in the regulation of bone formation came from the analysis of mouse models with impaired circadian regulation. These mice, which lack components of the molecular clock, namely the *Per* or *Cry* genes, display a high bone mass phenotype caused by increased bone

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formation (40). Moreover, they respond to intracerebroventricular infusion of leptin by a further increase of bone mass, suggesting that the components of the molecular clock are involved in the regulation of bone formation via the sympathetic nervous system. Interestingly, virtually all genes encoding members of the AP1-transcription factor family were expressed at higher levels in osteoblasts derived from mice lacking either the *Per* genes or *Adrb2,* the gene encoding the β2-adrenergic receptor (40). This increase was especially pronounced in the case of the *c-Fos* gene, whose expression can also be induced by the addition of isoprotenerol in wildtype osteoblasts. 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. Taken together, these data demonstrated that the expression of AP1-components is activated via sympathetic signaling, and that this induction is counteracted by the activity of clock gene products.

Conclusion

This brief overview illustrates how much progress has been made, yet it should not imply that we know everything about the transcriptional control of osteoblast differentiation. For instance, and to cite only one example famous in the field, we still have no experimental knowledge of how signals through the membrane receptor LRP5 affect bone formation. We also do not know much about the regulation of *Runx2* expression or whether different populations of osteoblast progenitors use different mechanisms to up-regulate *Runx2* expression. These are two of the challenges ahead of the field.

Conflict of Interest: The author reports that no conflict of interest exists.

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