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TRANSCRIPTION FACTORS AND OSTEOBLASTS

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1. ABSTRACT

Transcription factors play a key role in determination of the fate of the cells in osteoblastic and chondrocytic lineage. A runt family member, Cbfa, is indispensable for osteoblastic differentiation. Sox 9 and scleraxis are involved in the phenotypic expression in chondrocytes and the cells of early stage connective tissues. These transcription factors will give us a clue to unravel interaction of these known and yet unknown transcription factors to fully understand the mechanisms of skeletal cells ' differentiation and regulation of their functions.

2. INTRODUCTION

Osteoblasts are the cells which not only produce bone but act as a central coordinator to maintain balanced bone metabolism. Osteoblasts are derived from immature mesenchymal cells which could also give rise to chondrocytes, muscle cells, fat cells, ligament cells and tendon cells. This chapter will address the recent findings on the transcription factors which are critical in the determination of the fate of skeletal cells and the cells in their lineage.

Researches in the field of bone cells have received greater deal of public attention in the last decade than before due to the increasing number, and hence, significance of the fractures in elderly patients with osteoporosis in the modern aging society. Why should we learn about transcription factors to think about the treatment for bones in these patients who are mostly over 60's of age and hence most body cells are less vigorously proliferating and differentiating? The key issue is the fact that bone is not a "static" organ with regard to cell development such as brain, where cells with main function are already differentiated and do not proliferate or differentiate after the individuals reach their adulthood. Instead, bone is continuously rebuilt (remodeled) during the entire life including both the young and old generations. It is thought that whole bones in the body are totally replaced almost every several years. For this replacement, bone is on one hand being destroyed and on the other hand being newly made, meaning that osteoblasts proliferate and differentiate continuously regardless of the age. Similarly, osteoclasts are also continuously supplied by the differentiation of their blood-born precursor cells. This is why we believe that study on the nature of bone cell differentiation and proliferation would contribute to consider therapeutic measures for osteoporosis.

Bone is one of the organs contributing to the balance of body fluid calcium, which is the critical signaling molecule in a number of body functions. Approximately 300 to 500 mg calcium enters into bone and an equivalent amount is moving out from bone on a daily basis. About 99.9% of the total body calcium is stored in bone, and therefore, the above mentioned buffering capacity of bone serves as one of the internal "fail safe" mechanisms to maintain appropriate calcium concentration in body fluid and consequently this buffering function is one of the most life related critical functions of the body. This function is thought to have been established a long time ago when "life" moved from water to land. Skeletal cells including osteoblasts are responsible to build bone and to maintain calcium metabolism. In the following paragraphs, we will review the transcription factors which are operating to determine the production of bone forming cells.

3. CBFA1

In 1997, it was reported that PEBP2A1/Cbfa1 gene knock out mice completely lack osteoblasts (1, 2). This observation indicates that this transcription factor is prerequisite for osteoblastic differentiation. Striking features of the PEBP2A1/Cbfa1 knock out mice include not only a total lack of bone but also retardation in chondrocyte differentiation, reduction in the size and number of osteoclasts, and inability of osteoclasts to enter bone rudiments in order to resorb the part of cartilage.

There were several lines of different studies prior to the discovery of PEBP2A1/Cbfa1 to be the critical transcription factor for osteoblastic differentiation (3). Yoshiaki Ito's group was the first to report a family of

transcription factors which were identified during the studies on polyoma enhancer binding proteins and they were named as PEBPs. Speck 's group was studying the transcription factors which activate gene expression via binding to the core region of the moloney leukemia virus enhancer (CBF; core binding factor). However, the sequence of CBFA was the same as PEBP2 alpha1 and in this sense PEBP2 alpha was the first one to be discovered. Additionally, acute myeloblastic leukemia-associated gene (AML) was identified. Analysis of the sequences of these genes revealed that they all contain a domain homologous to the drosophila runt gene (4).

Common features of this group of transcription factors is that they form a heterodimer consisting of two subunits, alpha and beta, to be fully active. One component, the alpha subunit, binds to DNA directly but possess only weak transactivation activity, while the heterodimerization with the second component, beta, which by itself does not bind to DNA, enhances the transactivation activity to a full level. Three isoforms of the alpha subunit and one beta subunit have been found. The names of these components are corresponding to one another and from hereon, only one of the nomenclature will be used in this chapter, i.e. Cbfa1, 2, 3 for the DNA binding components, and Cbfb(PEBP2B) for the second component (5). A group of hematologists recently knocked out each of these genes to find out their functions.

Cbfb knock out mice (6) and Cbfa2 knocked-out mice are embryonic lethal due to the lack of hematopoiesis, and hemorrhage in brain. Cbfa1 knock out mice on the other hand are born alive, but die shortly after birth. Hematological analysis was conducted in the beginning and the researchers found only minor abnormality in blood cells, which could be possibly due to the secondary hematological changes induced by the lack of bone marrow. The examination of the newborn mice indicated that they were alive at least just after birth, but died due to the inability to breathe since they did not have bony ribs. Interestingly, alizarin red staining of the whole embryos shows the calcification of cartilage rudiments of tibiae and upper cervical vertebrae, indicating that calcification per se can take place in these mice (1).

Cbfa1 is expressed at high levels in normal osteoblasts (7) while it is also expressed at moderate levels in chondrocytes, tendon cells and T cells, suggesting other possible minor roles of Cbfa1 in these tissues. BMP (bone morphogenetic protein) treatment of the cells prepared from calvaria tissues of the Cbfa1 knock out mice was reported to enhance expression of alkaline phosphatase and osteocalcin indicating that these genes can be activated regardless of the absence of Cbfa1. During embryonic development, Cbfa1 mRNA levels in whole embryo are at their highest on day 12.5 d.p.c. (days post coitum) and then decline later on (7). This time point is several days earlier than the appearance of most of the bony tissues in the embryos.

In the Cbfa1 knock out embryos, alkaline phosphatase-positive cells are observed although the number of the cells is low, and low levels of type I collagen mRNA and alkaline phosphatase mRNA are expressed, indicating that early phases of osteoblastic differentiation may still take place in the absence of Cbfa-1 (1, 2). In contrast to type I collagen and alkaline phosphatase, expression of other phenotypic markers such as osteocalcin and osteopontin, which are relatively late differentiation markers of osteoblasts, is totally absent in these animals both at the protein level as well as message level. Introduction of Cbfa1 antisense fragments into rat osteosarcoma, ROS17/2.8 cells, could reduce the expression of osteocalcin, osteopontin, alkaline phosphatase and type I collagen. It appears, therefore, that this transcription factor acts, though not exclusively, at relatively late stages of osteoblastic differentiation.

Cbfa1 deficient mice derived cells express similar levels of cytokines and growth factors including BMPs and TGFbeta (transforming growth factor beta) compared to wild type cells. As these cells could still express osteocalcin when high doses of BMP7 was applied, the normal BMP levels in these cells appear to be insufficient to compensate the lack of Cbfa1. While BMP7 (7) or BMP4/7 specifically enhances expression of Cbfa-1 in MC3T3E1 (8), C3H10T1/2 and C2C12 cells, BMP2 does not enhance the Cbfa-1 expression indicating certain specificity. TGFbeta, retinoic acid, and FGF (fibroblast growth factor) do not largely change the levels of Cbfa-1, while glucocorticoids were resorted to suppress its expression. PTH (parathyroid hormone) was also reported to suppress Cbfa1 and expression of its target gene, collagenase type 3. Vitamin D interestingly suppresses Cbfa1 only in mice but not in rats and hence vitamin D suppresses osteocalcin in mice but not in rats.

At least two isoforms of Cbfa1 have been reported. One form, Cbfa1/Osf2 is specifically expressed in osteoblasts and this species, as reported by Ducy and Karsenty (7), contains a unique stretch of 87 amino acid at the N-terminal end. When this specific form of Cbfal (Cbfal/Osf2) is over-expressed, C3H10T1/2 cells express osteoblastic phenotype markers including alkaline phosphatase, type I collagen, osteopontin and osteocalcin. This Cbfa1/OSf2 is only expressed in bone amongst all tissues so far examined, at least by Northern blot analysis. The other isoform of Cbfa1, which has been known for a while as PEBP2alphaA/Cbfa1 or T cell type Cbfa1. When a region common to the two isoforms is used as a probe, expression was observed in many non-skeletal cells including T cells, embryonic cells, ES (embryonic stem) cells, NIH3T3 cells, C3H10T1/2 cells, and C2C12 cells (8). The mRNA levels detected by such Cbfa1/PEBP2alphaA probe in the above mentioned non-skeletal cells are almost similar to those in so-called skeletal cells such as MC3T3E1 and ROS17/2.8 cells as examined by Northern blot analysis using the probe which detects common regions of CBFA/PEBP2alphaA and Cbfa1/Osf2. Interestingly, overexpression of Cbfa-1/PEBP2alphaA suppressed expression of type I collagen in NIH3T3 and other nonskeletal cells. Even the levels of osteocalcin in ROS17/2.8 cells were slightly suppressed. Osteopontin levels in ROS17/2.8 cells were not altered by this overexpression; on the other hand, osteopontin expression in MC3T3E1 cells was enhanced by the overexpression of Cbfa1/PEBP2alphaA. These observations suggest that Cbfa1 could exert variable effects in the cells where it is

expressed depending on the sequences flanking the Cbfa1 binding sites, and hence depending on the other transcription factors which bind to these sites and possibly interact with these transcription factors. For instance, if Cbfa1 alone activates osteopontin mRNA, both Cbfa1/PEBP2alphaA and Cbfa1/Osf2 can simply activate osteopontin. On the other hand, if Cbfa-1 needs to interact via the extended 87 amino acids, with other transcription factors which bind to the sites located close to the Cbfa1 binding site, then overexpression of Cbfa-1/PEBP2alphaA which lacks the 87 amino acids stretch at its N-terminal may act as a dominant negative type inhibitor for the expression of the target gene. This point is still hypothetical and needs further investigation. These observations suggest that Cbfa1 plays a role not only in developmental stages of osteoblastic differentiation but also in the maintenance of the differentiated stages of bone metabolism. Interestingly, heterozygosity for a Cbfa1 mutation in humans causes cleidocranial dysplasia, and similar manifestations were found in mice (9, 10).

4. Sox9

Analysis of the tissue specific expression of the type II collagen gene identified a region in the intron from +2187 to +2234 which mediates cartilage tissue specific expression of this gene (11). Further analysis of the sequence showed that this region contains a consensus sequence for Sox. Sox9 has been shown to be expressed specifically in cartilage though not exclusively (12). It is also expressed in hair follicle. The sequence identified in the regulatory intron region of the type II collagen gene binds to Sox9 and mediates activation of gene transcription and hence it appears that Sox9 is at least involved in cartilage specific expression.

It is also reported that Sox9 is highly and specifically expressed in normal cartilage cells as well as chondrosarcoma derived cells. Sox9, expressed in yeast as a recombinant protein, can bind to Sox9 consensus sequence and overexpression can activate the intron fragment of the type II collagen gene as well as Sox9 site-linked reporter genes (13). Such sequences are also active *in vivo* and specifically mediate expression of reporter products such as lacZ in transgenic studies. It is, however, not yet known whether Sox9 alone can activate the expression of phenotype-related genes in both cartilage precursor cells or in already differentiated chondrocytes. Mutations of the transactivation domain or HMG (high mobility group) domain in Sox9 result in campomelic dysplasia, supporting its role in skeletogenesis. Sox9 knock out mice have not yet been reported and it will be intriguing to examine their phenotype and how it relates to campomelic dysplasia.

5. HELIX-LOOP-HELIX TYPE TRANSCRIPTION FACTORS

Helix-Loop-Helix type transcription factors (HLH-TFs) play crucial roles in determination of differentiation of muscle cells. Four myogenic determination factors (MDFs) including MyoD, Myf5, myogenin and MRF4 are acting in a hierarchy to process myogenic differentiation. These tissue specific HLH-TFs heterodimerize with ubiquitous HLH-TFs such as E12 and E47, to be fully active in binding to E-boxes which have been located in the target genes for MDFs and activate transcription. The third type HLH-TF, Id, lacks a DNA binding motif and therefore inactivates transcriptional activity of the other two types of HLH-TF mentioned above.

Osteoblasts and chondrocytes express this inhibitory type HLH-TF, Id, and its expression is suppressed by 1,25-dihydroxyvitamin D [1,25(OH)2D]. The Id gene promoter region contains a 57 basepairs (bp) sequence responsible for the 1,25(OH)2D suppression of Id gene expression. This region does not contain any known consensus sequence including hexamer half sites identified in the response elements of many nuclear receptor superfamily members. Instead, this sequence contains four repeats of a novel consensus sequence, which are all required for the mediation of vitamin D effects on the Id gene. Id gene is also under the control of bone morphogenetic protein (BMP), and glucocorticoids, indicating that at least a part of the regulation by these calciotropic hormones or cytokines could be mediated by the modulation of the transcriptional activity of the Helix-Loop-Helix type TFs. The osteocalcin promoter region contains an E-box sequence, located close to the CBFA binding site, and interaction of these different types of TFs might be responsible for finally determining the fate of the cells or differentiation.

Scleraxis was identified by two-hybrid screening of 10.5 day old mouse embryo-derived library using E12 as a bait. Scleraxis is also expressed at an early time point during embryonic development as well. It heterodimerizes with E12 and binds to E-box consensus to activate the reporter gene. However, its physiological function was not known.

Osteoblastic cells express modest levels of scleraxis and their levels are upregulated by TGFbeta. Scleraxis is also expressed in other types of connective tissue cells such as C2C12 and expression is down-regulated by BMPs in these cells. In a chondrocyte-like cell line, TC6 cells, scleraxis is expressed at relatively low levels and in this cell line as well, BMP down regulates and TGFbeta up-regulates scleraxis expression (15). These features indicate that scleraxis is the first basic HLH-type TF, which is positively regulated by the calciotropic factors.

Scleraxis overexpression in ROS17/2.8 cells suppresses the expression of the genes encoding osteoblastic phenotype-related proteins such as type I collagen and alkaline phosphatase. In these experiments, scleraxis enhances expression of cartilaginous phenotype-related genes, such as those encoding aggrecan and type II collagen. Analysis of the regulatory regions of the aggrecan gene identified a 27 bp response site, which we call AgE (16). This AgE sequence contains two tandem E-box sequences gapped by 3 bp. This sequence alone is sufficient and enough to confer responsiveness to scleraxis to a heterologous promoter such as the SV40 promoter. Within the entire 1kb region, only this E-box site was mutated to see its effect. This mutation totally abolishes the responsiveness of the 1kb region to scleraxis, indicating that the region is necessary and sufficient within the context of the aggrecan gene. Scleraxis binds the AgE sequence in gel shift assays and mutation of the E-box sequence specifically abolished the binding activity to scleraxis (16). These features suggest that scleraxis could be acting to promote expression of cartilage-related phenotypes, but also could possibly suppress expression of osteoblastic phenotypes. Such bi-directional characteristics have been similarly found in other HLH-TF such as MyoD. It is known that MyoD family

members interact with MEF2 to be fully active and it still remains to be solved whether scleraxis also requires partners in its action.

6. SUMMARY AND PERSPECTIVE

As reviewed in this chapter, multiple transcription factors are involved in skeletogenesis. Runt, Sox, and HLH family members have been found to be expressed in skeletal cells in the last couple of years. Further members of these transcription factor families or known or novel families of transcription factors, as well as the precise interactions amongst these factors should be identified to fully understand the mechanisms of skeletal cell differentiation. Such scientific bases should help develop measures to treat many bone diseases including osteoporosis.

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