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PROSPECTS

Regulation of Osteoblast Differentiation by Transcription Factors

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Abstract Runx2, osterix, and β-catenin are essential for osteoblast differentiation. Runx2 directs multipotent mesenchymal cells to an osteoblastic lineage, and inhibits them from differentiating into the adipocytic and chondrocytic lineages. After differentiating to preosteoblasts, β-catenin, osterix, and Runx2 direct them to immature osteoblasts, which produce bone matrix proteins, blocking their potential to differentiate into the chondrocytic lineage. Runx2 inhibits osteoblast maturation and the transition into osteocytes, keeping osteoblasts in an immature stage. Other transcription factors including Msx1, Msx2, Dlx5, Dlx6, Twist, AP1(Fos/Jun), Knox-20, Sp3, and ATF4 are also involved in osteoblast differentiation. To gain an understanding of bone development, it is important to position these transcription factors to the right places in the processes of osteoblast differentiation. J. Cell. Biochem. 99: 1233–1239, 2006.

Key words: Runx2; osterix; β-catenin; osteoblast; transcription factor

Skeletal component cells including osteoblasts, chondrocytes, adipocytes, myoblasts, tendon cells, and fibroblasts, are derived from mesenchymal stem cells. The lineages are determined by different transcription factors. The transcription factors, Runx2, osterix, and β-catenin, regulate osteoblast differentiation, Sox family transcription factors (Sox9, Sox5, and Sox6) regulate chondrocyte differentiation, MyoD transcription factors (MyoD, Myf5, and myogenin) regulate myogenic differentiation, and C/EBP family (C/EBPβ, C/EBPδ, and C/EBPα) and PPARγ2 transcription factors regulate adipocyte differentiation (Fig. 1).

The Runx family is composed of three genes, *Runx1/Cbfa2/Pebp2αB*, *Runx2/Cbfa1/Pebp2αA*, and *Runx3/Cbfa3/Pebp2αC*. All three genes contain a runt domain, which is the DNA-binding domain and is homologous with the *Drosophila* pair-rule gene runt. The Runx proteins form heterodimers with transcriptional co-activator core binding factor β (Cbfβ)/polyoma enhancer binding protein 2β (Pebp2β) in vitro and specifically recognize a consensus sequence, PyGPyGGTPy. Runx1 and Cbfβ are essential for hematopoietic stem cell differentiation. Runx2 is essential for osteoblast differentiation. Runx3 plays important roles in the growth regulation of gastric epithelial cells and in neurogenesis. Runx1 and Runx3 are also required for thymocyte development. Further, Runx2 and Runx3 are essential for chondrocyte maturation, which is a prerequisite for endochondral ossification. Cbfβ is also required for Runx2-dependent osteoblast and chondrocyte differentiation [Komori, 2005].

Osterix, which has three zinc finger motifs, belongs to the SP family transcription factors. Osterix$^{-/-}$ mice showed complete lack of osteoblasts, demonstrating that osterix is a second transcription factor that is essential for osteoblast differentiation [Nakashima et al., 2002]. Recently, the importance of Wnt signaling in bone formation was revealed. Wnts activate the canonical pathway by interacting with receptors of the Frizzled family and co-receptors of the LRP5/6 family. The canonical Wnt signaling

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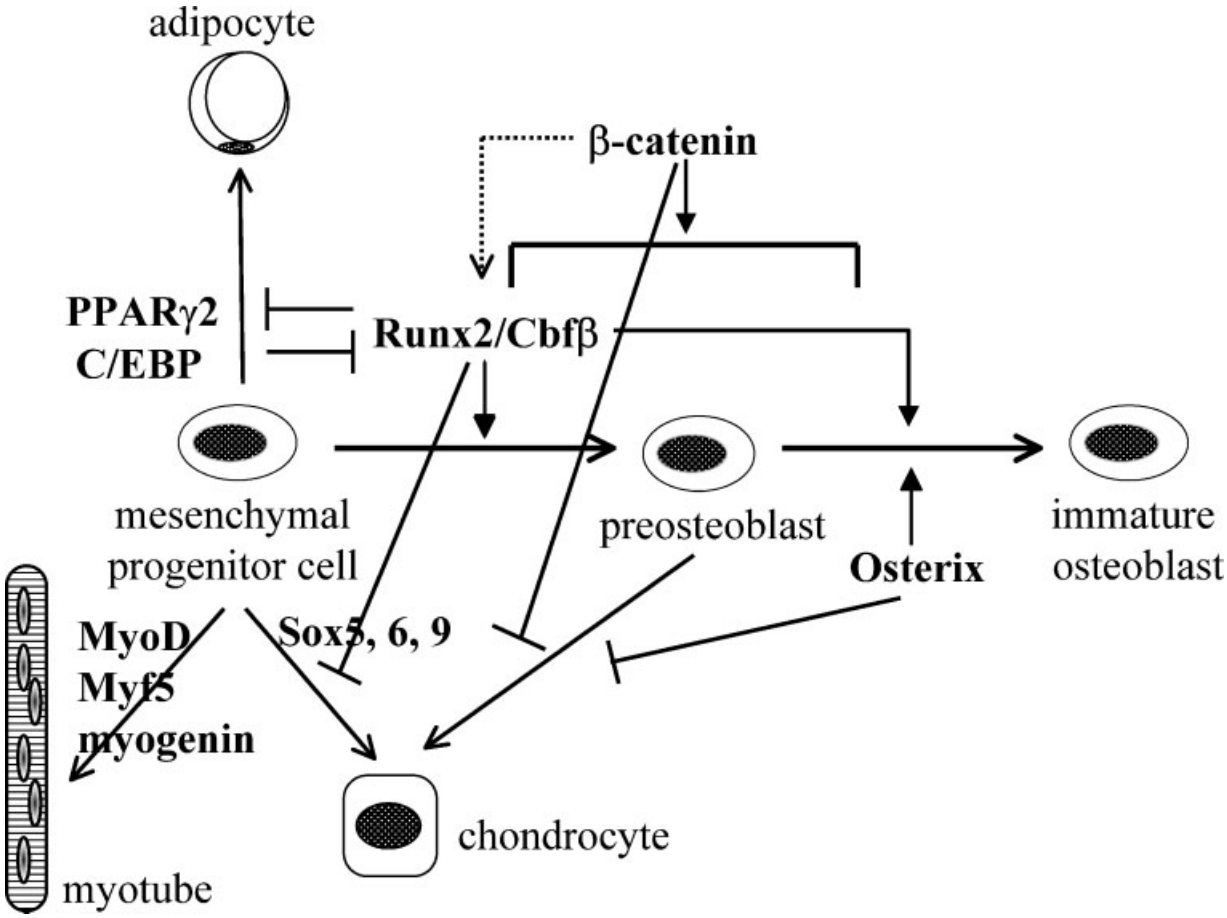

Fig. 1. Determination of osteoblastic lineage by transcription factors The differentiation of common mesenchymal progenitor cells into each skeletal component cells is determined by different transcription factors. In osteoblast differentiation, Runx2 directs mesenchymal progenitor cells to preosteoblasts, inhibiting adipocyte and chondrocyte differentiation. Runx2, β-catenin, and Osterix direct preosteoblasts to immature osteoblasts that express bone matrix protein genes, completely eliminating the potential to differentiate into chondrocytes.

pathway is transduced through stabilization of β-catenin protein molecules by inhibiting GSK-3-mediated β-catenin phosphorylation. Unphosphorylated β-catenin molecules accumulate in the cytoplasm, translocate to the nucleus, and activate the transcription of downstream genes by binding LEF/TCF transcription factors. Loss-of-function mutations in Lrp5 result in low bone mass, while gain-of-function mutations in Lrp5 result in high bone mass [Logan and Nusse, 2004]. Further, conditional deletion of β-catenin gene in Wnt1-Cre transgenic mice, in which Cre is expressed in neural crest cell precursors, results in loss of cranial bones derived from neural crest cells [Brault et al., 2001]. Finally, conditional deletion of β-catenin gene in Dermo-Cre or Prx1-Cre transgenic mice revealed an essential role of β-catenin in osteoblast differentiation [Day et al., 2005; Hill et al., 2005; Hu et al., 2005].

DETERMINATION OF AN OSTEOBLASTIC LINEAGE BY RUNX2, OSTERIX, AND β-CATENIN

Runx2$^{-/-}$ mice show complete lack of both intramembranous and endochondral ossification due to the absence of osteoblast differentiation [Komori et al., 1997; Otto et al., 1997]. Further, Runx2$^{-/-}$ calvarial cells spontaneously differentiate into adipocytes and differentiate into chondrocytes in the presence of BMP-2 in vitro, but they neither differentiate into osteoblasts even in the presence of BMP-2 in vitro nor in vivo. Therefore, Runx2$^{-/-}$ mesenchymal cells have the potential

to differentiate into adipocytes and chondrocytes [Kobayashi et al., 2000]. Runx2 is essential for differentiation of mesenchymal cells into osteoblasts and it inhibits their differentiation into adipocytes and chondrocytes. Osterix$^{-/-}$ mice also show the complete lack of both intramembranous and endochondral ossification due to the absence of osteoblast differentiation. Runx2 is expressed in the mesenchymal cells of osterix$^{-/-}$ mice, although osterix is not expressed in Runx2$^{-/-}$ mice. Therefore, osterix is a downstream gene of Runx2. In osterix$^{-/-}$ mice, perichondrial mesenchymal cells condensate and differentiate into chondrocytes, indicating that osterix$^{-/-}$ mesenchymal cells maintain the potential to differentiate into chondrocytes [Nakashima et al., 2002]. Inactivation of β-catenin in mesenchymal progenitor cells completely blocks osteoblast differentiation, and mesenchymal cells in the perichondrium and calvarium differentiate into chondrocytes [Day et al., 2005; Hill et al., 2005; Hu et al., 2005]. Therefore, β-catenin is essential for osteoblast differentiation and β-catenin$^{-/-}$ mesenchymal cells maintain their potential to differentiate into chondrocytes. As Runx2, but not osterix, is expressed in β-catenin$^{-/-}$ mesenchymal cells [Hill et al., 2005; Hu et al., 2005], β-catenin seems to be required for osteoblast differentiation at the preosteoblast stage. Further, β-catenin/TCF1 enhances Runx2 expression and Runx2 promoter activity [Gaur et al., 2005]. Thus, Runx2 directs mesenchymal progenitor cells to preosteoblasts, inhibiting their differentiation into adipocytes and chondrocytes, and β-catenin and osterix further direct the preosteoblasts to immature osteoblasts, completely eliminating the potential of preosteoblasts for differentiating to chondrocytes (Fig. 1).

Ihh is essential for osteoblast differentiation in endochondral bone, because Ihh$^{-/-}$ mice completely lack endochondral ossification due to the lack of osteoblasts. In Ihh$^{-/-}$ mice, Runx2 is expressed in chondrocytes but not in perichondrial cells which differentiate into osteoblasts in wild-type mice, indicating that Ihh is required for Runx2 expression in perichondrial cells. Runx2 and Runx3 are essential for chondrocyte maturation and induce Ihh expression in prehypertrophic chondrocytes, which in turn induces Runx2 expression in perichondrial cells. Further, no nuclear β-catenin was detected in the perichondrial cells of Ihh$^{-/-}$ mice, indicating that Ihh is required for canonical Wnt signaling in perichondrial cells [Hu et al., 2005]. Therefore, there is a linkage between chondrocyte maturation and osteoblast differentiation in endochondral ossification, and Ihh mediates the linkage [Komori, 2005].

REGULATION OF OSTEOBLAST DIFFERENTIATION BY RUNX2

Mesenchymal stem cells differentiate into immature osteoblasts, which express bone matrix protein genes, through the actions of Runx2, osterix, and β-catenin. The immature osteoblasts, which express high levels of osteopontin, differentiate into mature osteoblasts, which express high levels of osteocalcin, and finally the mature osteoblasts are embedded in the bone matrix to become osteocytes. Overexpression of type II Runx2, whose protein sequence starts with the sequence, MASNS, encoded by exon 1, in osteoblasts using the 2.3-kb Col1a1 promoter, caused osteopenia with fractures in mice [Liu et al., 2001; Geoffroy et al., 2002]. In these mice, osteoblast maturation and the transition of osteoblasts to osteocytes are severely inhibited. The DNA-binding sites of Runx2 in major bone matrix protein genes including the Col1a1, osteopontin, bone sialoprotein, and osteocalcin genes, have been identified, and Runx2 induced the expression of these genes or activated their promoters in vitro [Komori, 2005]. However, overexpression of type II Runx2 in osteoblasts severely reduced osteocalcin expression [Liu et al., 2001], despite the finding that Runx2 strongly induced osteocalcin expression in mouse embryonic fibroblast cell line C3H10T1/2 [Ducy et al., 1997; Harada et al., 1999]. Further, overexpression of type I Runx2, whose protein sequence starts with the sequence, MRIPVD, encoded by exon 2, also inhibited osteoblast maturation and the transition into osteocytes. However, the degree of inhibition of osteoblast maturation and the transition to osteocytes were much milder in type I Runx2 transgenic mice than in type II Runx transgenic mice [Kanatani et al., 2006]. Therefore, these in vivo findings combined with the previous in vitro data indicate that Runx2 triggers the expression of major bone matrix protein genes at an early stage of osteoblast differentiation, leading to the cells acquiring an osteoblastic phenotype but

maintaining an osteoblastic cells in an immature stage. Therefore, Runx2 plays an important role in maintaining a supply of immature osteoblasts (Fig. 2).

REGULATION OF RUNX2 AND OSTERIX FUNCTION BY OTHER TRANSCRIPTION FACTORS AND CO-REGULATORS

Many molecules interact with Runx2 and enhance or inhibit Runx2 functions. Cbfβ is the most important co-regulator of Runx2 and is required for DNA binding of Runx2. Although Cbfβ is required for Runx2-dependent bone development, osteoblast differentiation occurs to a limited degree in $Cbfb^{-/-}$ mice that express the Cbfb transgene under the control of Gata1 promoter [Yoshida et al., 2002]. Recently, we examined the function of Cbfβ in postnatal bone development and found that Cbfβ regulates Runx2 function isoform-dependently [Kanatani et al., 2006]. Type II Runx2, but not type I Runx2, seems to be able to exert its function to a limited degree in the absence of Cbfβ. Other unknown factors may substitute for Cbfβ in the DNA binding of and transcriptional activation by type II Runx2. Several transcription factors including C/EBPβ, C/EBPδ, ETS1, Menin, Smad1, and Smad5, interact with Runx2 and enhance the transcriptional activity of Runx2. Grg5, Rb, TAZ, and p204 interact with Runx2 and function as transcriptional co-activators of Runx2 [Komori, 2005; Liu et al., 2005]. Other transcription factors and co-regulators including C/EBPδ, Dlx3, Msx2, PPARγ, Twist, Stat1, Smad3, Yes, and TLE, reduce the transcriptional activity of Runx2 [Kang et al., 2005; Komori, 2005]. Little is known about factors that interact with osterix. Recently, it was shown that NFATc1 interacts with osterix and enhances Col1a1 promoter activity [Koga

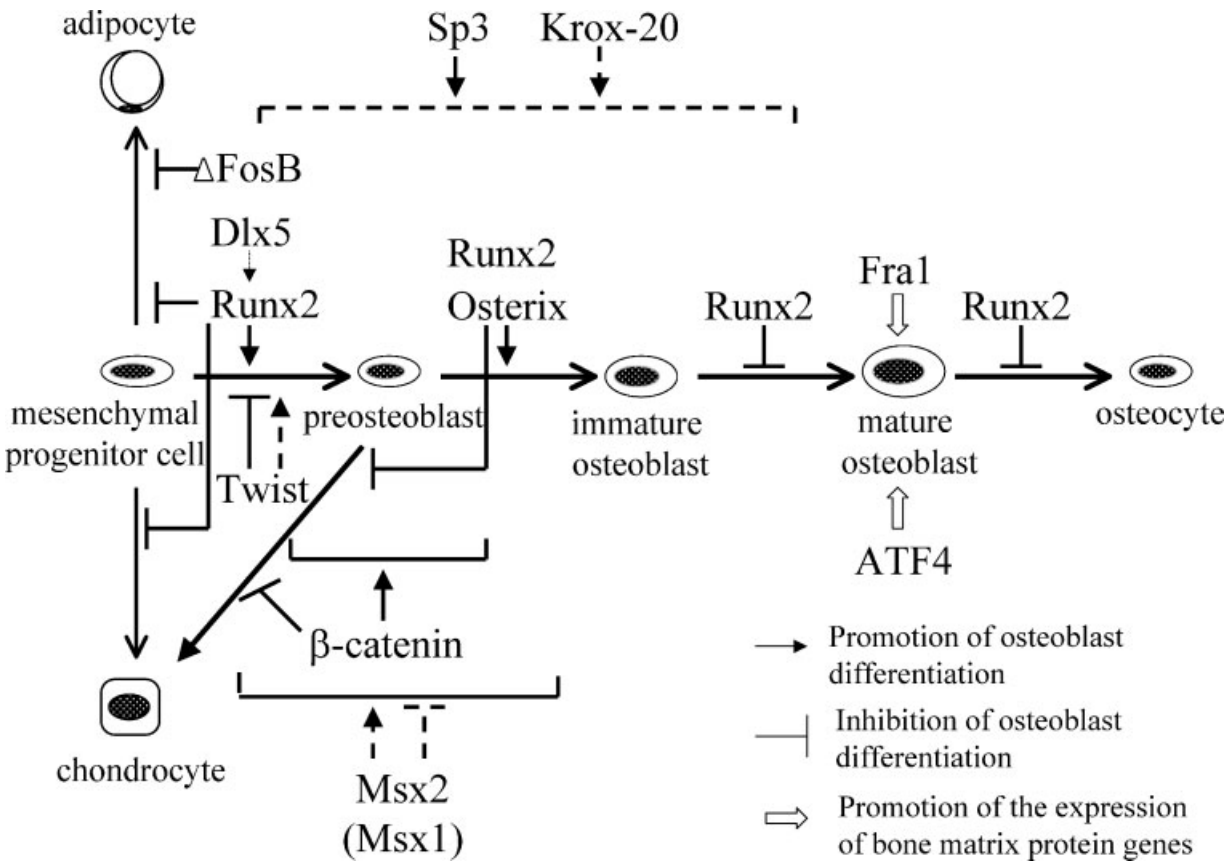

Fig. 2. Regulation of osteoblast differentiation by transcription factors. Besides Runx2, Osterix, and β-catenin, many transcription factors are involved in osteoblast differentiation. Twist proteins interact with Runx2 and inhibit Runx2 function. However, Twist and Msx2 co-operatively promote osteoblast differentiation. Some studies reported that Msx2 enhances osteoblast differentiation, while other studies reported that Msx2 inhibits it. Msx2 was also reported to promote osteoblast proliferation. The involvement of Msx1 in osteoblast differentiation is limited in comparison with Msx2. The dotted lines indicate that the physiological function or the stage at which the factor mainly works remains to be proven.

et al., 2005]. The physiological importance of many of these interactions in bone development remains to be elucidated.

REGULATION OF OSTEOBLAST DIFFERENTIATION BY OTHER TRANSCRIPTION FACTORS

Msx1 and Msx2 are transcription factors that belong to homeobox proteins. $Msx1^{-/-}$ mice show a delay in cranial bone formation, indicating that Msx1 is involved in osteoblast differentiation in the cranial bone. $Msx2^{-/-}$ mice also show a delay in cranial bone formation. Parietal foramina in humans is caused by haploinsufficiency of Msx2. In $Msx1^{-/-}Msx2^{-/-}$ mice, cranial bone formation is severely inhibited, indicating that Msx1 and Msx2 have a redundant function in cranial bone formation [Satokata et al., 2000]. The function of Msx2 in osteoblasts is still controversial. One study suggested that Msx2 inhibits differentiation of osteoblast precursors and immature osteoblasts, resulting in an increase in the source of osteoblastic cells [Liu et al., 1999]. However, other studies demonstrated that Msx2 promotes osteoblast differentiation and/or proliferation [Ishii et al., 2003; Ichida et al., 2004] (Fig. 2).

Dlx5 and Dlx6 are transcription factors that belong to homeobox proteins. Dlx5 is expressed in osteoblasts in an entire skeleton; however, the abnormalities in $Dlx5^{-/-}$ mice are restricted to craniofacial bones. In $Dlx5^{-/-}Dlx6^{-/-}$ mice, there are severe abnormalities in craniofacial bone formation, and the calvarium, maxilla, and mandibula are not formed. Further, loss or fusion of the central digits in the forepaws and hindpaws of the mutant mice was observed. Similar abnormalities are observed in patients with split-hand/split-foot malformation (SHFM). Ossification in the limbs, vertebrae, and ribs is apparently retarded in an embryonic stage, indicating that Dlx5 and Dlx6 are involved in endochondral ossification [Robledo et al., 2002]. However, chondrocyte maturation is also retarded in $Dlx5^{-/-}Dlx6^{-/-}$ mice, which leads to the delay in osteoblast differentiation, although Dlx5 and Dlx6 have been reported to be expressed in perichondrial cells but not in chondrocytes [Robledo et al., 2002]. Therefore, their direct effects on osteoblasts in endochondral bone remain to be clarified. In vitro studies showed that Dlx5 specifically regulates the expression and promoter activity of type II Runx2 [Lee et al., 2005] (Fig. 2).

Twist proteins (Twist-1 and Twist-2) are basic helix-loop-helix (bHLH)-containing transcription factors. Mutation of the Twist-1 gene was identified in patients with Saethre–Chotzen syndrome, which is an autosomal dominant disease characterized by premature closure of cranial sutures and limb abnormalities. An open fontanelle, which is a characteristic of cleidocranial dysplasia, is seen in $Runx2^{+/-}$ mice, but not in $Runx2^{+/-}Twist\text{-}1^{+/-}$ mice. Twist proteins interact with Runx2 and inhibit osteoblast differentiation at an early stage [Bialek et al., 2004]. However, $Twist^{+/-}$ mice also show an open frontal suture, and this feature is enhanced in $Twist^{+/-}Msx2^{+/-}$ mice, indicating that Twist and Msx2 co-operatively induce the differentiation and proliferation of the frontal bone skeletogenic mesenchyme [Ishii et al., 2003] (Fig. 2).

AP1 (Fos/Jun) plays important roles in bone formation. Overexpression of either ΔFosB or Fra1 results in osteopetrosis due to enhanced bone formation. An osteopetrosis in transgenic mice overexpressing ΔFosB is due to an increased number of osteoblasts and inhibition of differentiation into an adipocytic lineage at an early stage of mesenchymal cell differentiation [Kveiborg et al., 2004]. Further, conditional $Fra1^{-/-}$ mice show reduced expression of matrix proteins including osteocalcin, MGP, and type II collagen, in bone and cartilage, indicating that Fra1 plays an important role in matrix production [Eferl et al., 2004] (Fig. 2).

Krox-20 and SP3 are transcription factors that have Zn fingers. In $Krox\text{-}20^{-/-}$ mice, endochondral ossification is disturbed and formation of the trabecular bone is reduced [Levi et al., 1996]. As Krox-20 is expressed in terminally differentiated chondrocytes as well as osteoblasts, the function of Krox-20 in endochondral ossification remains to be clarified. $SP3^{-/-}$ mice show a delay in both intramembranous and endochondral ossification. Further, bone nodule formation is reduced in $SP3^{-/-}$ ES cells, suggesting that SP3 is involved in osteoblast differentiation [Gollner et al., 2001] (Fig. 2).

ATF4 is a basic leucine-zipper transcription factor and belongs to the CREB family. $ATF4^{-/-}$ mice show a delay in ossification and osteopenia. ATF4 is a substrate of RSK2 (ribosomal

serine/threonine kinase 2). Patients with Coffin–Lowry syndrome, which is an X-linked disease characterized by mental retardation, delay in bone age, delay in closure of the fontanelle, and short stature, have mutations in RSK2. Both ATF4$^{-/-}$ mice and RSK2$^{-/-}$ mice show disturbance in type I collagen production [Yang et al., 2004] (Fig. 2). Further, it has been shown that ATF4 interacts with Runx2 and enhances the expression and promoter activity of osteocalcin [Xiao et al., 2005].

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