Skeletal Development Through the Regulation of Chondrocyte and Osteoblast Differentiation by Runx2

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ABSTRACT

Runx2 is a transcription factor that belongs to Runx family (Runx1, Runx2, and Runx3). Runx2 interacts with many other transcription factors and co-regulators in the transcriptional regulation of its target genes. Cbfb is one of the co-regulators, forms heterodimers with Runx2, and is required for Runx2-dependent transcriptional regulation. Runx2 is essential for the commitment of multipotent mesenchymal cells into the osteoblastic lineage, because Runx2-deficient mice show complete lack of bone formation due to the absence of osteoblasts. Further, Runx2 inhibits adipocyte differentiation, because Runx2-deficient calvarial cells spontaneously differentiate into adipocytes. Overexpression of Runx2 in osteoblasts inhibits osteoblast maturation but decreased the expression of major bone matrix protein genes. Therefore, Runx2 triggers the gene expression of bone matrix proteins, while keeping the osteoblastic cells in an immature stage. Moreover, Runx2 strongly inhibits the transition of osteoblasts into osteocytes. Runx2 and Runx3 double knockout mice showed that Runx2 and Runx3 have redundant functions in chondrocytes, and that they are essential for chondrocyte maturation. Runx2 directly induces Ihh expression and coordinates the proliferation and differentiation of chondrocytes. Therefore, Runx2 regulates bone formation by regulating osteoblast differentiation as well as chondrocyte maturation.

Key words: Runx2, chondrocyte differentiation, osteoblast differentiation, Runx3, Cbfb

The vertebrate skeleton is composed of cartilage and bone. Bone is formed through either intramembranous or endochondral ossification. Osteoblasts directly form intramembranous bones, while chondrocytes first form a cartilaginous skeleton which is then replaced with bone by osteoblasts and osteoclasts through the process of endochondral ossification. These processes are regulated by many factors, and specific transcription factors play essential roles in the differentiation of chondrocytes and osteoblasts. Runx2 plays essential roles in chondrocyte maturation and osteoblast differentiation (Komori 2005). Runx2 belongs to Runx family (Runx1/Cbfa2/Pebp2aB, Runx2/Cbfa1/Pebp2aA and Runx3/Cbfa3/Pebp2aC). All three genes contain a runt domain, which is the DNA-binding domain and is homologous with the Drosophila pair-rule gene runt. Runx1 is essential for hematopoietic stem cell differentiation (Komori and Kishimoto, 1998). Runx3 plays important roles in the growth regulation of gastric epithelial cells and in neurogenesis, and Runx3-deficient ($Runx3^{-/-}$) mice show inflammatory bowel disease and eosinophilic lung inflammation (Li *et al.*, 2002; Inoue *et al.*, 2002; Levanon *et al.*, 2002; Brenner *et al.*, 2004; Fainaru *et al.*, 2004). Cbfb, which has no DNA binding domain, forms heterodimers with Runx proteins. Recently, we found that Cbfb is required for Runx2-dependent osteoblast differentiation and chondrocyte maturation (Yoshida *et al.*, 2002). Mutations in RUNX genes have been linked to human diseases. Aberrant translocations of RUNX1 are responsible for acute myeloid leukemia. Heterozygous mutations in the RUNX2 gene cause cleidocranial dysplasia, a pathological condition characterized by hypoplastic clavicles, opened fontanelles, supernumerary teeth and short stature. RUNX3 misfunction has been shown to be related to gastric cancer (Komori *et al.*, 2002).

Runx2 and chondrocyte differentiation

In $Runx2^{-/-}$ mice, whose entire skeleton is composed of cartilage, chondrocyte differentiation is severely disturbed throughout most of the skeleton (Inada *et al.*, 1999). Runx2 promotes chondrocyte maturation (Enomoto *et al.*, 2000; Ueta *et al.*, 2001), overexpression of dn-Runx2 inhibits chondrocyte maturation (Ueta *et al.*, 2001). Interestingly, most of the joints are fused in transgenic mice that overexpress Runx2, and the majority of chondrocytes in dn-Runx2 transgenic mice retain a marker of chondrocytes in the permanent cartilage (Ueta *et al.*, 2001). Therefore, Runx2 is required for chondrocyte maturation and is involved in determining whether chondrocytes acquire a permanent or transient phenotype (Fig. 1).

Although chondrocyte maturation is inhibited in $Runx2^{-/-}$ mice, chondrocyte maturation to the terminal stage eventually occurs in the restricted skeleton, indicating that other factors are also involved in chondrocyte maturation. All Runx genes are expressed in chondrocytes (Levanon *et al.*, 2001; Stricker *et al.*, 2002), and transgenic mice that overexpress dn-Runx2 under the control of the Col2a1 promoter/enhancer, in which the functions of all Runx proteins in chondrocytes are inhibited, show more severe inhibition of chondrocyte maturation than that seen in $Runx2^{-/-}$ mice (Uete *et al.*, 2001). These find-

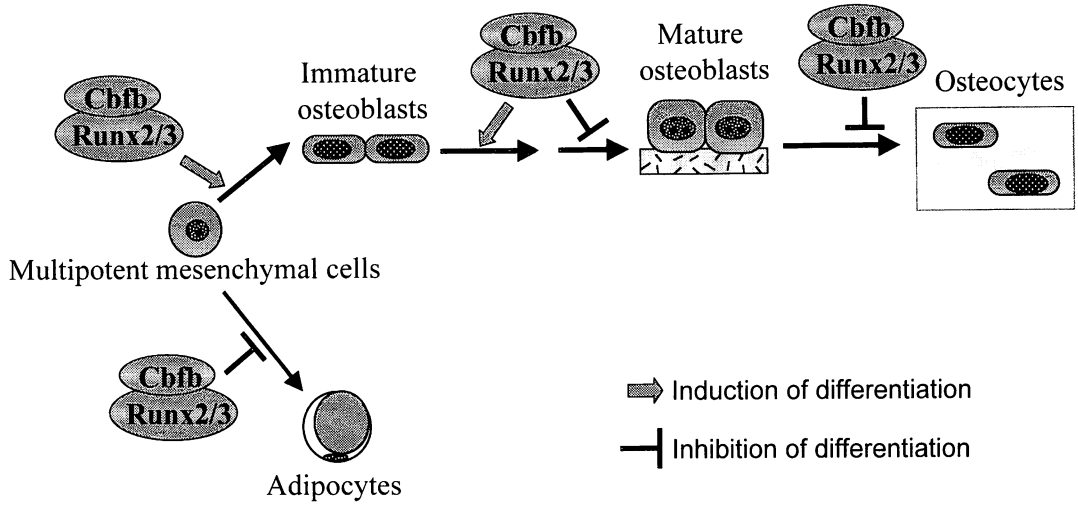

Fig. 1 Regulation of chondrocyte maturation and proliferation by Runx2 and Runx3.
Runx2 and Runx3 are essential for chondrocyte maturation and inhibit chondrocytes from acquiring the phenotype of permanent cartilage. Cbfb is required for the Runx2-dependent chondrocyte maturation. Runx2 directly induces Ihh expression, which enhances chondrocyte proliferation and inhibits chondrocyte maturation through PTHrP. With regard to Runx3, either Runx3 induces Ihh expression indirectly or additional factors are required for Ihh induction by Runx3.

ings indicate that Runx1 and/or Runx3 is involved in chondrocyte maturation. Runx1$^{-/-}$ mice are embryonic lethal at E12.5 due to the absence of hematopoiesis. Runx3$^{-/-}$ mice die after birth probably due to the abnormality in gastric epithelial cells. We tried to find the function of Runx1 and Runx3 in chondrocyte differentiation by mating Runx1$^{+/-}$ mice with Runx2$^{+/-}$ mice or by mating Runx3$^{+/-}$ mice with Runx2$^{+/-}$ mice. Runx1$^{+/-}$Runx2$^{-/-}$ mice showed a similar disturbance of chondrocyte maturation as compared with Runx2$^{-/-}$ mice, indicating that the contribution of Runx1 to chondrocyte maturation, if any, seems to be limited. In contrast, Runx2$^{-/-}$Runx3$^{+/-}$ mice showed that the chondrocyte maturation is more severely inhibited than Runx2$^{-/-}$ mice, indicating that Runx3 is also involved in chondroxyte maturation. We finally got Runx2$^{-/-}$Runx3$^{-/-}$ mice in which chondrocyte maturation in the entire skeleton was completely inhibited. It demonstrates that Runx2 and Runx3 are essential for chondrocyte maturation (Yoshida *et al.*, 2004) (Fig. 1).

Interestingly, the length of the limbs of Runx2$^{-/-}$ mice was reduced and further reduced in Runx2$^{-/-}$3$^{-/-}$ mice. The reduced chondrocyte proliferation was the cause of shortened limbs in the Runx2$^{-/-}$ mice, and the small volume of the cells in the diaphyses was the cause of the further reduction of limb length in Runx2$^{-/-}$Runx3$^{-/-}$ mice. Runx2 directly induces the expression of Ihh, which is a positive regulator of chondrocyte proliferation (Yoshida *et al.*, 2004). As Ihh is also a negative regulator of chondrocyte maturation (Vortkamp *et al.*, 1996), Runx2 and Runx3 coordinate chondrocyte maturation and proliferation and regulate limb growth through the induction of Ihh (Fig. 1). Further, Pthlh inhibits Runx2 expression through the PKA signaling pathway (Iwamoto *et al.*, 2003; Li *et al.*, 2004). As Ihh induces Pthlh expression (Vortkamp *et al.*, 1996), Runx2 expression is also regulated by a negative feedback loop (Fig. 1).

Runx2 and osteoblast differentiation

Runx2$^{-/-}$ mice completely lack bone formation due to the absence of osteoblasts, demonstrating that Runx2 is essential for osteoblast differentiation (Komori *et al.*, 1997) (Fig. 2). Runx2$^{-/-}$ calvarial cells fail to differentiate into osteoblasts both in vitro and in vivo, even in the presence of BMP-2. However, in vitro studies showed that Runx2$^{-/-}$ calvarial cells spontaneously differentiate into adipocytes, and they differentiate into chondrocytes in the presence of BMP-2. Therefore, Runx2$^{-/-}$ mesenchymal cells completely lack the ability to differentiate into osteoblasts, but retain the capacity to differentiate into adipocytes and chondrocytes (Kobayashi *et al.*, 2000). Furthermore, Runx2$^{-/-}$ chondrocytes also spontaneously differentiated into adipocytes, and introduction of Runx2 prevented adipocyte differentiation, indicating that Runx2 inhibits adipogenesis (Enomoto *et al.*, 2004).

Many recent in vitro studies demonstrated that Runx2 is a positive regulator that up-regulates the expression of or activates the promoters of genes related to bone matrix proteins including Col1a1, Col1a2, osteopontin, bone sialoprotein (BSP), osteocalcin, fibronectin, MMP13, and OPG (Komori 2002). Further, the overexpression of dominant negative (dn)-Runx2 under the control of the osteocalcin promoter, which directs reporter gene expression to mature osteoblasts, results in severe osteopenia due to drastic reductions in the expression of genes encoding the main bone matrix proteins including Col1a1, Col1a2, osteopontin, BSP, and osteocalcin (Ducy *et al.*, 1999). However, transgenic mice that overexpressed Runx2 under the control of a 2.3-kb mouse Col1a1 promoter, which specifically directs reporter gene expression to osteoblasts, showed osteopenia with multiple fractures (Liu *et al.*, 2001). Most of the osteoblasts of these mice exhibited less mature phenotypes, and the numbers of

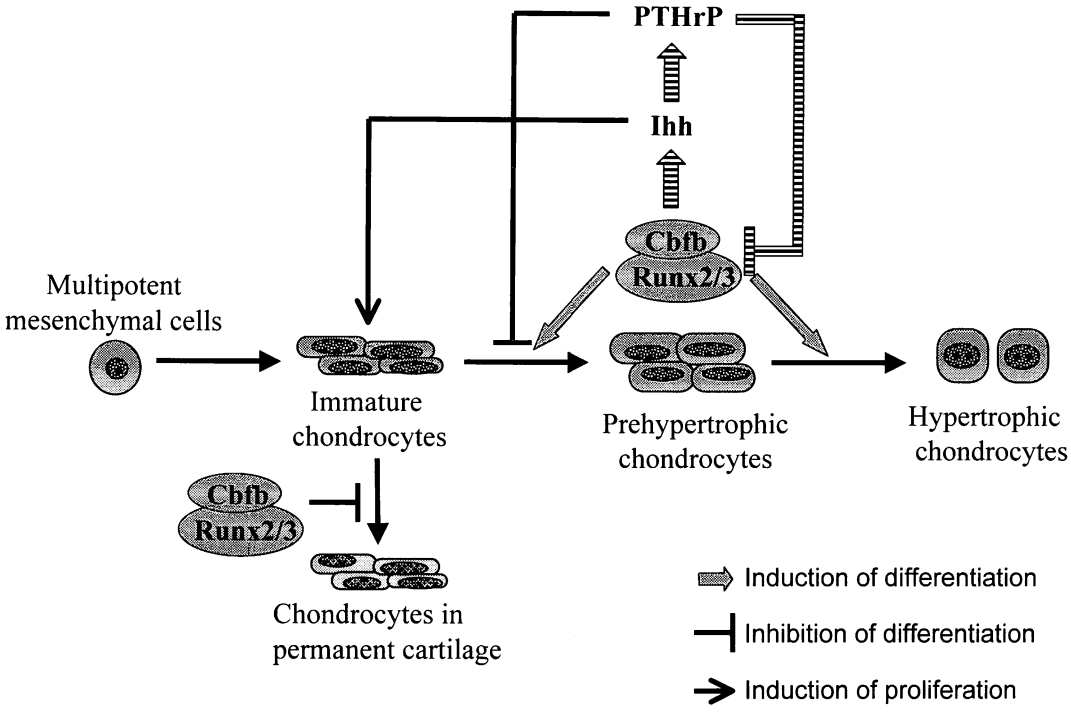

Fig. 2 Regulation of osteoblast differentiation by Runx2
Runx2 induces mesenchymal condensation, inhibits their differentiation into adipocytes, induces the expression of osteoblastic markers, and allows the mesenchymal progenitor cells to differentiate into the osteoblastic lineage. Cbfb is required for the Runx2-dependent osteoblast differentiation. However, Runx2 inhibits osteoblast differentiation at a late stage and severely inhibits the transition of osteoblasts into osteocytes.

terminally differentiated osteoblasts, which strongly express osteocalcin, and osteocytes were greatly diminished. As a result, in the osteoblasts of these mice, the expression of Col1a1, alkaline phosphatase, osteocalcin, and MMP13, all of which normally increase during osteoblast maturation, were reduced. Further, osteocytes were severely reduced in these mice. These findings indicate that Runx2 inhibits osteoblast differentiation at a late stage (Fig. 2). Our recent studies on dn-Runx2 transgenic mice with high transgene expression under the control of the same 2.3-kb mouse Col1a1 promoter showed that major bone matrix protein gene expression was not significantly affected by the suppression of Runx2 function in mature osteoblasts (manuscript in preparation). Therefore, there is a controversy in the function of Runx2 in the regulation of bone matrix protein genes. In the dn-Runx2 transgenic mice generated by Ducy *et al.* (1999), the level of transgene expression was very weak and was similar to the level of endogenous Runx2 expression, and expression of the transgene was transiently detected only at 2-4 weeks of age. To explain the drastic phenotype caused by the weak expression of dn-Runx2, they showed that dn-Runx2 binds the Runx2 binding sites at unusually strong affinity. However, we compared the affinity to the Runx2 consensus oligonucleotides between Runx2 and dn-Runx2 and found that dn-Runx2 binds Runx2 binding sites at the similar affinity with native Runx2, indicating that high expression of dn-Runx2 is required for the dominant negative effect (manuscript in preparation). These findings along with the in vitro data, indicate that Runx2 induces the expression of major bone matrix protein genes in osteoblast progenitors, allowing the cells to acquire the osteoblastic phenotype while keeping the osteoblastic cells in an immature stage (Fig. 2).

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