Regulation of Skeletal Development by the Runx Family of Transcription Factors

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Abstract   The Runx (runt-related protein) family of transcription factors plays important roles in different tissues and cell lineages. Runx1 determines commitment to the hematopoietic cell lineage and Runx2 determines commitment to the osteoblastic lineage. Cbfβ is required for Runx1- and Runx2-dependent transcriptional regulation. Runx2 interacts with many other transcription factors and co-regulators in the transcriptional regulation of its target genes. Runx2 is essential for the commitment of multipotent mesenchymal cells into the osteoblastic lineage and inhibits adipocyte differentiation. Runx2 induces the gene expression of bone matrix proteins, while keeping the osteoblastic cells in an immature stage. Runx2 and Runx3 have redundant functions in chondrocytes, and they are essential for chondrocyte maturation. Runx2 directly induces Indian hedgehog (Ihh) expression and co-ordinates the proliferation and differentiation of chondrocytes. Therefore, elucidation of the signaling pathways through Runx2 and Runx3 will unravel the complex mechanism of skeletal development. J. Cell. Biochem. 95: 445–453, 2005.   © 2005 Wiley-Liss, Inc.

Key words: Runx2; Cbfa1; Runx3; Cbfb; osteoblast; chondrocyte; Ihh

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. The transcription factor Sox9 plays an essential role in mesenchymal condensation leading to formation of the cartilaginous template; Sox5 and Sox6 are required for the production of cartilaginous matrix; and runt-related protein 2 (Runx2)/ core binding factor 1 (Cbfa1)/polyoma enhancer binding protein 2αA

(Pebp2αA) plays an important role in the maturation of chondrocytes, which is a prerequisite for endochondral ossification. In osteoblast differentiation, Runx2 and Osterix play essential roles in the commitment of pluripotent mesenchymal cells to the osteoblastic lineage [Komori, 2002].

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 [Komori and Kishimoto, 1998]. Runx1 and Cbfβ are 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 [Inoue et al., 2002; Levanon et al., 2002; Li et al., 2002; Brenner et al., 2004;

Fainaru et al., 2004]. Further, Runx1 and Runx3 are required for thymocyte development [Taniuchi et al., 2002]. Mutations in these genes result in specific diseases. Specific translocations in the *Runx1* and *Cbfb* genes are involved in acute myeloid leukemia. Haploinsufficiency of *Runx2* causes cleidocranial dysplasia, while *Runx3* has been suggested to be related to gastric cancer [Komori, 2002].

Recently, it was reported that Runx3 also plays an important role in chondrocyte maturation [Yoshida et al., 2004]. This review focuses on the involvement of the Runx family of transcription factors in skeletal development.

REGULATION OF THE EXPRESSION AND FUNCTION OF Runx2

Runx2 is transcriptionally upregulated by bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs) and retinoic acid, and downregulated by $1\alpha,25(OH)_2D_3$ and tumor necrosis factor-α (TNF-α). TGF-β upregulates or downregulates *Runx2* transcription depending on the cell lines [Komori, 2002] (Fig. 1). Further, the regulatory region of *Runx2* has multiple Runx2 binding sites, and Runx2 negatively regulates its own promoter in osteoblast precursors [Drissi et al., 2000; Yoshida et al., 2002]. However, Runx2 has also been shown to positively regulate the activity of its own promoter [Ducy et al., 1999; Geoffroy et al., 2002]. The main factors that regulate *Runx2* expression differ depending on the time and location during the differentiation of osteoblasts and chondrocytes.

Runx2 is post-translationally regulated by hormones (Fig. 1). Estrogen enhances the transcriptional activity of Runx2, and estrogen receptor α directly interacts with Runx2 [Sasaki-Iwaoka et al., 1999; McCarthy et al., 2003]. Parathyroid hormone (PTH) induces phosphorylation of the transactivation domain of Runx2 during the process of matrix metalloproteinase 13 (MMP13) activation [Selvamurugan et al., 2000]. A glucocorticoid inhibited insulin-induced chondrogenesis of ATDC5 cells by suppressing PI3K-Akt signaling as well as DNA binding of Runx2 and Runx2-dependent transcription [Fujita et al., 2004a]. The reduced Runx2 activity was likely caused by the suppressed PI3K-Akt signaling, because PI3K-Akt
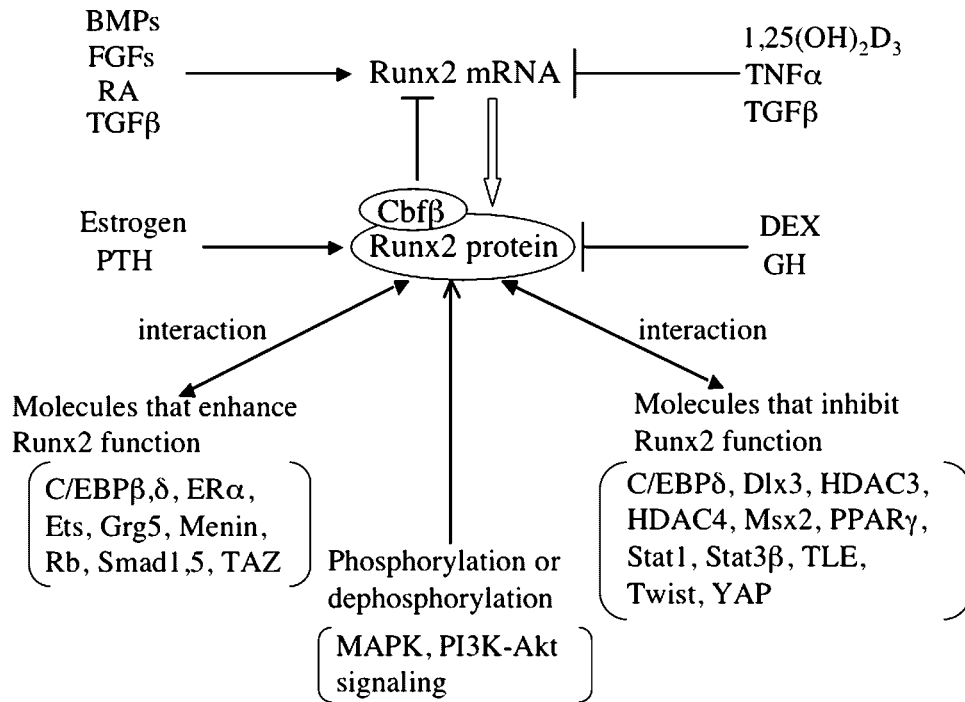

Fig. 1. Regulation of Runx2 expression and function Various molecules affect *Runx2* expression at the transcriptional level. At the post-translational level, hormones and molecules that interact with Runx2 affect Runx2 function. Various signals induce the phosphorylation or dephosphorylation of Runx2 or DNA-binding complex molecules containing Runx2, modifying Runx2 function. Runx2/Cbfβ heterodimers negatively regulate the *Runx2* promoter. RA, retinoic acid; DEX, dexamethasone; GH, growth hormone.

signaling activates Runx2 function [Fujita et al., 2004b]. Growth hormone inhibits the transcriptional activity of Runx2 by enhancing the interaction of Runx2 and Stat3β [Ziros et al., 2004].

Many molecules interact with Runx2 and enhance or inhibit Runx2 function (Fig. 1). C/EBPβ and δ interact with Runx2 and synergistically activate the osteocalcin promoter [Gutierrez et al., 2002]. Runx2 and ETS1 markedly enhance osteopontin promoter activity in a synergistic manner [Sato et al., 1998]. Menin, the product of the multiple endocrine neoplasia type 1 (*MEN1*) gene, interacts with Runx2 and enhances the transcriptional activity of Runx2 [Sowa et al., 2004]. The interaction of Runx2 with Smad1 or 5 enhances the transcriptional and osteogenic activities of Runx2 [Zhang et al., 2000; Nishimura et al., 2002]. Grg5, Rb, and TAZ interact with Runx2 and function as transcriptional coactivators of Runx2 [Thomas et al., 2001; Cui et al., 2003; Wang et al., 2004].

C/EBPδ also functions as a negative regulator, and the binding of Runx2 with C/EBPδ suppresses the ability of Runx2 to drive C/EBPδ expression [McCarthy et al., 2000]. Dlx3, Msx2 and PPARγ interact with Runx2 and inhibit the transcriptional activity of Runx2 on the osteocalcin promoter [Shirakabe et al., 2001; Jeon et al., 2003; Hassan et al., 2004]. Histone deacetylase (HDAC) 4 suppresses chondrocyte maturation by inhibiting Runx2 function, and HDAC3 interacts with Runx2 and suppresses the expression of Runx2 target genes [Schroeder et al., 2004; Vega et al., 2004]. Stat1 in its transcriptionally latent form interacts with Runx2 in the cytoplasm of osteoblasts, inhibiting the nuclear localization of Runx2 [Kim et al., 2003]. Twist interacts with the DNA-binding domain of Runx2 and transiently inhibits Runx2 function at an early stage of osteoblast differentiation [Bialek et al., 2004]. Src/Yes signaling leads to the formation of Runx2-Yes transcriptional complexes that attenuate Runx2 transcriptional activity [Zaidi et al., 2004]. TLE interacts with the C terminus of Runx proteins, thereby repressing osteocalcin transcription [Javed et al., 2000]. Indeed, Runx2 affects the function of other transcription factors. Runx2 contributes to the $1\alpha,25(OH)_2D_3$-dependent enhancement of the osteocalcin promoter [Paredes et al., 2004]. Therefore, many transcription factors and co-regulators interact with Runx2, modulating its function at many steps. However, the physiological importance of many of these interactions in skeletal development remains to be elucidated.

The phosphorylation or dephosphorylation of Runx2 and DNA-binding complex molecules containing Runx2 seems to be another mechanism through which Runx2 function is modulated (Fig. 1). MAPK pathways activate and phosphorylate Runx2 [Xiao et al., 2000, 2002; Qiao et al., 2004]. In contrast, serine phosphorylation of Runx2 negatively alters Runx2 function [Wee et al., 2002]. PI3K-Akt signaling enhances the DNA binding and transcriptional activity of Runx2 without modulating the phosphorylation of Runx2 [Fujita et al., 2004b]. Therefore, PI3K-Akt signaling may regulate the DNA-binding activity of Runx2 by inducing the phosphorylation of a molecule(s) that forms a DNA-binding complex with Runx2 or by inducing the dephosphorylation through the activation of phosphatase. As the time course of the inhibition of the DNA-binding activity of Runx2 upon treatment with LY294002 or U01126 differed [Fujita et al., 2004b], the mechanism of the phosphorylation or dephosphorylation of DNA-binding complex molecules containing Runx2 induced by PI3K-Akt signaling should be different from that induced by the MAPK pathway. Finally, the cAMP pathway, which is a major intracellular pathway mediating PTH signals, suppresses Runx2 through proteolytic degradation of Runx2 itself that involves a ubiquitin/proteasome-dependent mechanism [Tintut et al., 1999].

REQUIREMENT OF Cbfb FOR SKELETAL DEVELOPMENT

$Cbfb^{-/-}$ mice die at E11.5–13.5 due to the absence of fetal liver hematopoiesis and hemorrhaging in the central nervous system, indicating that Cbfβ is essential for the emergence of hematopoietic stem cells [Komori and Kishimoto, 1998]. However, the role of Cbfβ in skeletal development remained to be clarified, and it has been studied by three different approaches. Miller et al. [2002] rescued the hematopoiesis of $Cbfb^{-/-}$ mice by introducing *Cbfb* using the *Tek/Tie2* promoter, which directs the transgene expression to endothelial cells, a fraction of hematopoietic stem cells and committed hematopoietic progenitors. Yoshida et al. [2002] rescued the hematopoiesis of $Cbfb^{-/-}$

mice by introducing *Cbfb* using the *Gata1* promoter, which directs transgene expression to erythroid and megakaryocytic lineages. Although the degree of the rescue of hematopoiesis in individual lineages differed, both procedures successfully prolonged the survival of *Cbfb*$^{-/-}$ mice until birth. In these mice (*Cbfb*$^{-/-}$ tg mice), both intramembranous and endochondral ossification were severely inhibited. A limited degree of osteoblast differentiation occurred, and chondrocyte maturation was severely delayed in these mice. Kundu et al. [2002] developed knock-in mice that expressed a fusion protein of Cbfβ containing the first 151 amino acids of Cbfβ and green fluorescent protein. In this mouse model, hematopoiesis was normal during development, but skeletal development was defective. Both intramembranous and endochondral ossification were inhibited but less severely than in the previous two models. Using *Cbfb*$^{-/-}$ tg calvarial cells, it was shown that Cbfβ is essential for DNA binding of Runx2 and Runx2-dependent transcriptional activation [Yoshida et al., 2002]. Thus, Cbfβ is required for the function of Runx2 in osteoblast differentiation and chondrocyte maturation.

Further, in *Cbfb*$^{-/-}$ tg calvarial cell cultures, osteoblast differentiation was not observed even though *Runx2* expression was upregulated throughout the culture period, indicating that Runx2 negatively regulates its own promoter in osteoblastic precursor cells under normal conditions and that this activity is dependent on the presence of Cbfβ [Yoshida et al., 2002]. This finding is consistent with the results of *Runx2* promoter analysis [Drissi et al., 2000]. In contrast to Runx1, the Runx2 protein was stable in the absence of Cbfβ in vivo [Huang et al., 2001; Yoshida et al., 2002]. The upregulation of *Runx2* expression and the stability of the Runx2 protein in *Cbfb*$^{-/-}$ tg mice may explain the less severe delay in the skeletal development seen in *Cbfb*$^{-/-}$ tg mice compared with *Runx2*$^{-/-}$ mice [Yoshida et al., 2002].

OSTEOGENESIS AND Runx2

Runx2$^{-/-}$ mice and *Osterix*$^{-/-}$ mice completely lack bone formation due to the absence of osteoblasts, demonstrating that both Runx2 and Osterix are essential for osteoblast differentiation [Komori et al., 1997; Otto et al., 1997; Nakashima et al., 2002]. Mesenchymal condensation in the perichondrial region is observed in *Osterix*$^{-/-}$ mice, but not in *Runx2*$^{-/-}$ mice. *Osterix* expression is absent in *Runx2*$^{-/-}$ mice, although *Runx2* is normally detected in *Osterix*$^{-/-}$ mice [Nakashima et al., 2002]. These findings indicate that Runx2 is required for mesenchymal condensation in osteoblast differentiation, and that the *Osterix* gene is downstream of the *Runx2* gene (Fig. 2). Further, Twist transiently inhibits Runx2 function during the early stage of osteoblast differentiation [Bialek et al., 2004]. In *Osterix*$^{-/-}$ embryos, the expression of *Sox9*, *Sox5*, *Coll2a1*, *Indian hedgehog* (*Ihh*), and *Col10a1*, which are markers of chondrocytes, is observed in the mesenchymal condensation of the perichondrial region, indicating that *Osterix*$^{-/-}$ mesenchymal cells that express *Runx2* have the ability to differentiate into chondrocytes [Nakashima et al., 2002]. *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]. In contrast, PPARγ inhibits osteogenesis and physically interacts with Runx2, leading to suppression of Runx2 function [Lecka-Czernik et al., 1999; Jeon et al., 2003; Akune et al., 2004]. These findings suggest that Runx2 and PPARγ inhibit each other's functions. Thus, in the lineage determination of skeletal component cells, both Runx2 and Osterix play essential roles in the commitment of multipotent mesenchymal cells to the osteoblastic lineage, and Runx2 inhibits adipogenesis while Osterix inhibits chondrogenesis (Fig. 2).

Many recent in vitro studies demonstrated that Runx2 is a positive regulator that upregulates 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 expres-
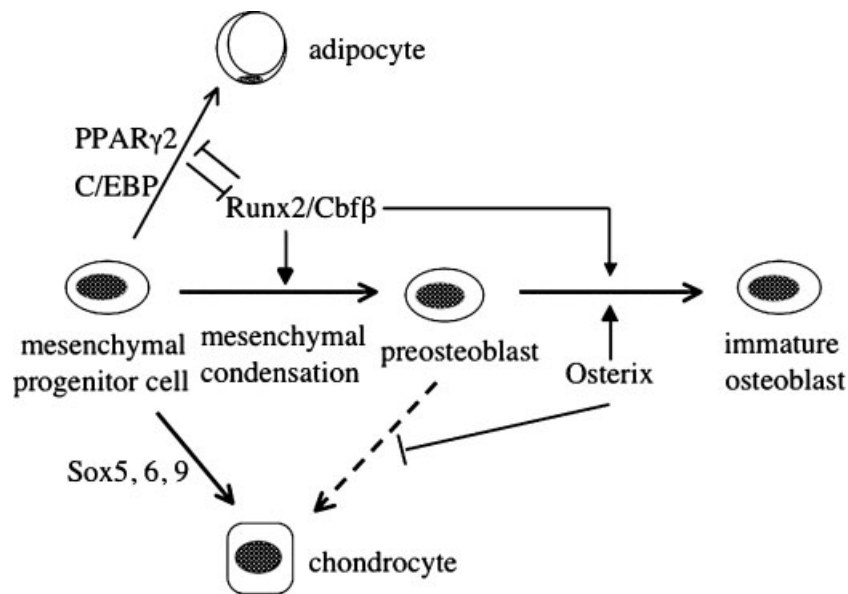
Fig. 2. Commitment of mesenchymal cells to the osteoblast lineage. Runx2 induces mesenchymal condensation, inhibits their differentiation into adipocytes, induces the expression of osteoblastic markers, and allows the mesenchymal progenitor cells to differentiate into the osteoblastic lineage. Osterix further allows the preosteoblasts to differentiate into immature osteoblasts by inhibiting their differentiation into chondrocytes. Osterix is probably also involved in induction of the expression of osteoblastic markers.

sion to mature osteoblasts, results in 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; Geoffroy et al., 2002]. Most of the osteoblasts of these mice exhibited less mature phenotypes, and the numbers of 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. These findings indicate that Runx2 inhibits osteoblast differentiation at a late stage. Our recent studies on dn-*Runx2* transgenic mice 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). It was reported that Runx2 regulates the transcription of *ATF4*, which is

required for terminal differentiation of osteoblasts, *osteocalcin* expression, and type I collagen synthesis [Yang et al., 2004]. As Runx2 inhibits the terminal differentiation of osteoblasts, the physiological significance of Runx2-dependent regulation of *ATF4* expression needs to be further investigated. 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.

There are two Runx2 isoforms with different N-termini, type I and type II Runx2, and these isoforms have different functions in osteoblast differentiation and bone matrix protein gene expression [Harada et al., 1999]. Although both isoforms are expressed in osteoblasts as well as chondrocytes, type I *Runx2* is also expressed in osteoprogenitors and other mesenchymal tissues [Enomoto et al., 2000; Banerjee et al., 2001; Choi et al., 2002]. Type II $Runx2^{-/-}$ mice were recently generated [Xiao et al., 2004]. Both intramembranous and endochondral ossification occurred in these mice, but less effectively than in wild-type mice. These findings indicate that both type I and type II Runx2 are involved in osteoblast differentiation and
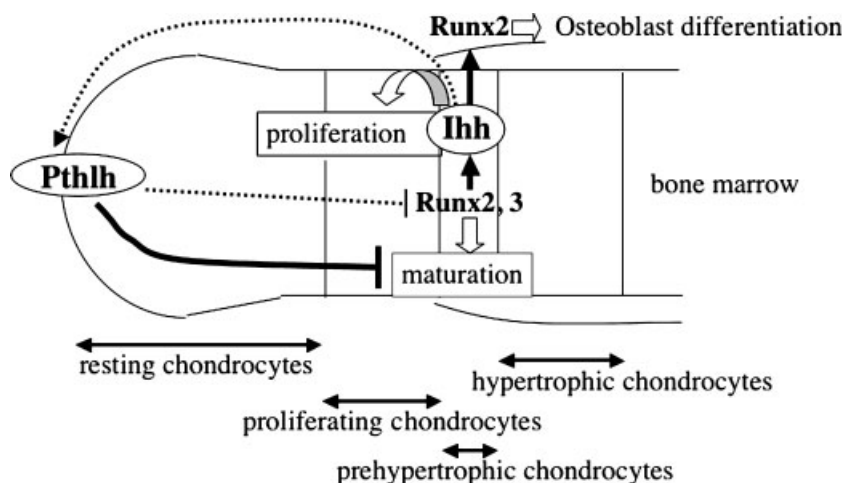
Fig. 3. Regulation of chondrocyte maturation and proliferation by Runx2 and Runx3. Runx2 and Runx3 are essential for chondrocyte maturation. Runx2 directly induces *Ihh* expression, which enhances chondrocyte proliferation directly and inhibits chondrocyte maturation through Pthlh. With regard to Runx3, either Runx3 induces *Ihh* expression indirectly or additional factors are required for *Ihh* induction by Runx3. Ihh is essential for *Runx2* expression in the perichondrial region; however, the mechanism of *Runx2* induction remains to be clarified.

chondrocyte maturation. In these mice, the zone of hypertrophic chondrocytes was expanded and the primary trabeculae were poorly formed. These features may be due to impaired vascular invasion into the cartilage, because type II *Runx2* is strongly expressed in terminally differentiated chondrocytes and vascular invasion is impaired in $Runx2^{-/-}$ cartilage [Enomoto et al., 2000; Himeno et al., 2002].

ROLES OF Runx2 AND Runx3 IN 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; Kim et al., 1999]. Runx2 promotes chondrocyte maturation [Enomoto et al., 2000; Takeda et al., 2001; Ueta et al., 2001; Stricker et al., 2002], overexpression of dn-*Runx2* inhibits chondrocyte maturation [Ueta et al., 2001; Stricker et al., 2002] and overexpression of *Runx2* in the chondrocytes of $Runx2^{-/-}$ mice restores chondrocyte maturation [Takeda 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. As to regulation of the expression of *Col10a1*, which is a hypertrophic chondrocyte-specific marker, Runx2 upregulated the 4-kb *Col10a1* promoter in MCT cells [Zheng et al., 2003]. As Runx2 does not upregulate *Col10a1* in $Runx2^{-/-}$ chondrocytes [Yoshida et al., 2004], however, additional factors seem to be required for *Col10a1* expression. HDAC4 suppresses chondrocyte maturation through the inhibition of Runx2 [Vega et al., 2004].

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 [Ueta et al., 2001]. These findings indicate that Runx1 and/or Runx3 is involved in chondrocyte maturation. In $Runx2^{-/-}3^{-/-}$ mice, chondrocyte maturation in the entire skeleton is completely inhibited. In these mice, all chondrocytes express *Col2a1* but not PTH/Parathyroid hormone-related

peptide (*Pthlh*) receptor (*Pthr1*), *Ihh* nor *Col10a1*, demonstrating that Runx2 and Runx3 are essential for chondrocyte maturation [Yoshida et al., 2004]. The degree of maturational delay of the chondrocytes in $Runx1^{+/-}2^{-/-}$ mice was the same as that in $Runx2^{-/-}$ mice (Yoshida and Komori, unpublished data), whereas the maturational delay of the chondrocytes in $Runx2^{-/-}3^{+/-}$ mice was more severe than that in $Runx2^{-/-}$ mice, indicating that the contribution of Runx1 to chondrocyte maturation, if any, seems to be limited. The length of the limbs of $Runx2^{-/-}3^{-/-}$ mice is severely reduced due to reduced chondrocyte proliferation and small volume of the cells in the diaphyses. Runx2 directly induces the expression of *Ihh*, which is a positive regulator of chondrocyte proliferation [St-Jacques et al., 1999]. However, with regard to Runx3, either Runx3 induces *Ihh* expression in an indirect manner or additional factors are required for *Ihh* induction by Runx3 [Yoshida et al., 2004]. As Ihh is also a negative regulator of chondrocyte maturation [Vortkamp et al., 1996], Runx2 and Runx3 co-ordinate chondrocyte maturation and proliferation and regulate limb growth through the induction of *Ihh* (Fig. 3). 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. 3). Although Ihh is required for *Runx2* expression in the perichondrial region [St-Jacques et al., 1999], the mechanism of the induction of *Runx2* expression remains to be clarified (Fig. 3).

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