Roles of Runx2 in Skeletal Development

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Abstract

Runx2 is the most upstream transcription factor essential for osteoblast differentiation. It regulates the expression of *Sp7*, the protein of which is a crucial transcription factor for osteoblast differentiation, as well as that of bone matrix genes including *Spp1*, *Ibsp*, and *Bglap2*. Runx2 is also required for chondrocyte maturation, and Runx3 has a redundant function with Runx2 in chondrocyte maturation. Runx2 regulates the expression of *Col10a1*, *Spp1*, *Ibsp*, and *Mmp13* in chondrocytes. It also inhibits chondrocytes from acquiring the phenotypes of permanent cartilage chondrocytes. It regulates chondrocyte proliferation through the regulation of *Ihh* expression. Runx2 enhances osteoclastogenesis by regulating *Rankl*. Cbfb, which is a co-transcription factor for Runx family proteins, plays an important role in skeletal development by stabilizing Runx family proteins. In Cbfb isoforms, Cbfb1 is more potent than Cbfb2 in Runx2-dependent transcriptional regulation; however, the expression level of *Cbfb2* is three-fold higher than that of *Cbfb1*, demonstrating the requirement of Cbfb2 in skeletal development. The expression of *Runx2* in osteoblasts is regulated by a 343-bp enhancer located upstream of the P1 promoter. This enhancer is activated by an enhanceosome composed of Dlx5/6, Mef2, Tcf7, Ctnnb1, Sox5/6, Smad1, and Sp7. Thus, Runx2 is a multifunctional transcription factor that is essential for skeletal development, and Cbfb regulates skeletal development by modulating the stability and transcriptional activity of Runx family proteins.

Keywords

Runx2 • Osteoblast • Chondrocyte • Cbfb • Enhancer

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6.1 Introduction

Skeletal component cells including osteoblasts, chondrocytes, adipocytes, myoblasts, tendon cells, and fibroblasts, are derived from mesenchymal stem cells. Their lineages are determined by different transcription factors. Runx2 and Sp7 regulate osteoblast differentiation, the Sox family (Sox9, Sox5, and Sox6) regulates chondrocyte differentiation, the MyoD family (MyoD, Myf5, and myogenin) regulates myogenic differentiation, and the C/EBP family (C/EBPβ, C/EBPδ, and C/EBPα) and PPARγ2 regulate adipocyte differentiation (Komori 2006).

Bony skeletons are formed through intramembranous and endochondral ossification. In intramembranous bone development, mesenchymal cells differentiate into osteoblasts and bone is directly formed by osteoblasts. In endochondral ossification, cartilaginous skeletons are formed by chondrocytes, which acquire mature phenotypes at the diaphysis, in which vascular invasion occurs, and osteoclasts and mesenchymal cells invade the cartilage. Terminally differentiated chondrocytes die due to apoptosis, mesenchymal cells differentiate into osteoblasts, and bone is formed on the rudiments of cartilaginous structures. Cartilaginous structures are then completely replaced with bone (Inada et al. 1999; Marks Jr. and Odgren 2002).

Runx2, which belongs to the Runx family of proteins consisting of Runx1, Runx2, and Runx3, is a transcription factor that is essential for skeletal development. Runx family proteins have a runt domain, which directly binds to DNA. Runx2 is known to form a heterodimer with Cbfb and acquires an enhanced DNA-binding capacity (Komori 2005). A heterozygous mutation of RUNX2 has been shown to cause cleidocranial dysplasia, which is characterized by hypoplastic clavicles, open fontanelles, supernumerary teeth, and a short stature (Mundlos et al. 1997). In this review, a focus has been placed on the functions of Runx2 and Cbfb in the regulation of osteoblast and chondrocyte differentiation as well as transcriptional regulation of the *Runx2* gene.

6.2 Roles of Runx2 in Osteoblast Differentiation

Osteoblasts are completely absent in *Runx2*$^{-/-}$ mice, which indicates that Runx2 is an essential transcription factor for osteoblast differentiation (Komori et al. 1997; Otto et al. 1997) (Fig. 6.1). Canonical Wnt signaling and Sp7 are also crucial for osteoblast differentiation (Komori 2006). After committing to the osteoblastic lineage, osteoblasts express bone matrix protein genes at different expression levels depending on the maturation level of the cells. Immature mesenchymal cells and preosteoblasts weakly express *Col1a1*, the expression of which is up-regulated in immature osteoblasts (Inada et al. 1999). Immature osteoblasts have been shown to express *Spp1* and then *Ibsp*, while mature osteoblasts strongly express *Bglap2* (Maruyama et al. 2007; Aubin and Triffitt 2002). Mature osteoblasts are embedded into the bone matrix and ultimately become osteocytes, which express *Dmp1* (Toyosawa et al. 2001). Previous studies demonstrated that the expression of bone matrix protein genes including *Spp1*, *Ibsp*, and *Bglap2*, is virtually absent in *Runx2*$^{-/-}$ mice (Komori et al. 1997; Inada et al. 1999). Runx2 has the ability to up-regulate the expression of bone matrix protein genes including *Col1a1*, *Spp1*, *Ibsp*, *Bglap*, and *Fn1* (fibronectin 1) (Ducy et al. 1997; Sato et al. 1998; Harada et al. 1999; Lee et al. 2000), and Runx2 activates reporter activities including *Col1a1*, *Col1a2*, *Spp1*, and *Bglap2* promoters (Banerjee et al. 1997; Harada et al. 1999; Jimenez et al. 1999; Sato et al. 1998; Kern et al. 2001). However, the expression of *Ibsp* is reduced by Runx2 and HDAC3 *in vitro*, and Runx2 represses *Ibsp* promoter activity (Javed et al. 2001; Lamour et al. 2007).

The function of Runx2 in the early stage of osteoblast differentiation is very clear because osteoblast marker gene expression is absent in *Runx2*$^{-/-}$ mice, indicating that Runx2 is essential for the differentiation of mesenchymal stem cells into osteoblasts in an early stage (Komori et al. 1997; Otto et al. 1997) (Fig. 6.1). However, the

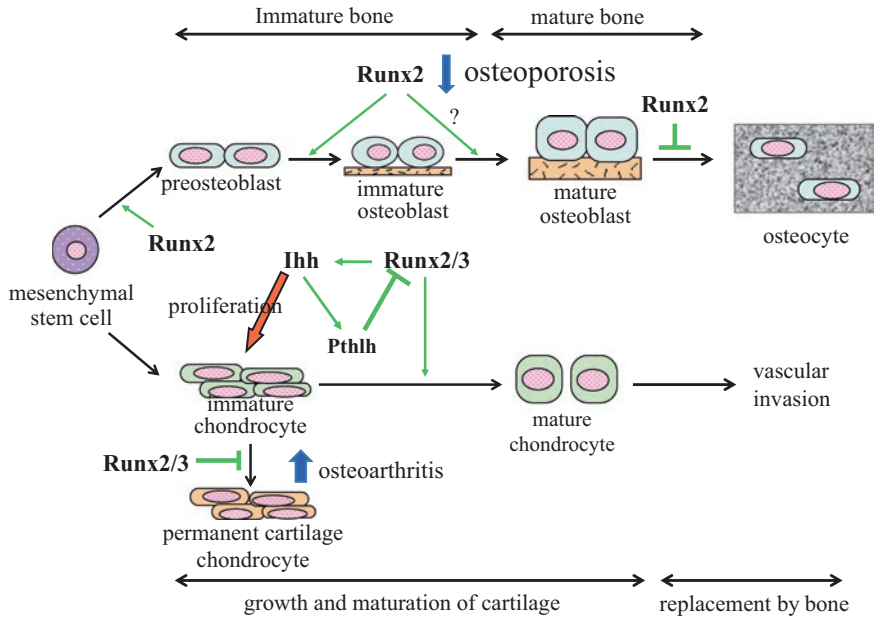

Fig. 6.1 Functions of Runx2 in osteoblast and chondrocyte differentiation. Runx2 directs the differentiation of mesenchymal stem cells to preosteoblasts and further differentiation to immature osteoblasts. The functions of Runx2 in committed osteoblasts are controversial. Runx2 inhibits the transition of osteoblasts to osteocytes. Although Runx2 is not required for the differentiation of mesenchymal stem cells to immature chondrocytes, it is necessary for the maturation of immature chondrocytes. Runx3 has a redundant function in chondrocyte maturation. Runx2 inhibits chondrocytes from acquiring the phenotypes of permanent cartilage chondrocytes. It also regulates chondrocyte proliferation by regulating *Ihh* expression. Ihh up-regulates the expression of *Pthlh*, the protein of which inhibits Runx2 and chondrocyte maturation. Pathologically, reductions in Runx2 expression and activity in the osteoblast lineage are associated with osteoporosis, while the up-regulation of Runx2 expression and activity in permanent cartilage chondrocytes is related to osteoarthritis

functions of Runx2 in committed osteoblasts are controversial. *Runx2* conditional knockout mice using Cre transgenic mice under the control of a 2.3-kb *Col1a1* promoter, which directs transgene expression to committed osteoblasts, were recently reported by two groups. The conditional deletion of exon 4, which contains a part of the runt domain, results in no overt phenotypes (Takarada et al. 2013), whereas mice with the conditional deletion of exon 8, which produces a truncated Runx2 protein, have been shown to develop osteopenia due to reduced bone formation (Adhami et al. 2015). Osteoclastogenesis is also reduced in the latter mice. Since these studies used the same Cre transgenic line, the expression level of the Cre transgene does not appear to be the cause of the difference in these phenotypes. However, the genetic backgrounds of their mice differed, which may explain the discrepancies observed. Further investigations are needed in order to clarify the functions of Runx2 in committed osteoblasts (Fig. 6.1).

We and others previously reported that the overexpression of *Runx2* using a 2.3-kb *Col1a1* promoter resulted in osteopenia due to reduced bone formation (Liu et al. 2001; Geoffroy et al. 2002; Kanatani et al. 2006). The expression of Runx2 is initially detected in preosteoblasts, increases in immature osteoblasts, and then decreases during osteoblast maturation. It is strongly expressed in embryos and young mice after birth, but gradually decreases and is low in adult mice (Maruyama et al. 2007). Therefore, the phenotypes of *Runx2* transgenic mice indicate that the maintenance of the strong expression of *Runx2* inhibits osteoblast maturation and keeps the osteoblasts in an immature stage. Furthermore, osteocytes are virtually absent in

Runx2 transgenic mice, indicating that Runx2 inhibits the transition of osteoblasts to osteocytes (Liu et al. 2001) (Fig. 6.1). Runx2 has also been shown to induce the expression of *Rankl*, which is essential for osteoclast differentiation, and enhances bone resorption (Enomoto et al. 2003; Geoffroy et al. 2002).

Cortical bone is reduced in dominant-negative (dn) *Runx2* transgenic mice under the control of a 2.3-kb *Col1a1* promoter. Bone formation in trabecular bone is marginally reduced in young adult dn-*Runx2* transgenic mice, but trabecular bone increases by 7 months of age. A previous study demonstrated that mineralization is increased in trabecular bone, urinary deoxypyridinoline, which is a marker for bone resorption, is reduced in dn-*Runx2* transgenic mice, and ovariectomy increases bone resorption in wild-type mice, but not in dn-*Runx2* transgenic mice (Maruyama et al. 2007). Therefore, osteoblast maturation appears to be accelerated in dn-*Runx2* transgenic mice and leads to the formation of mature bone, which is resistant to bone resorption, because cortical bone formed by mature osteoblasts is more resistant to bone resorption than trabecular bone formed by relatively immature osteoblasts. Furthermore, dn-*Runx2* inhibits osteoclastogenesis *in vitro*. Therefore, Runx2 regulates bone maturity and osteoclastogenesis and is involved in bone reductions after an estrogen deficiency (Maruyama et al. 2007).

6.3 Roles of Runx2 in Chondrocyte Differentiation

Although the entire skeleton of *Runx2*$^{-/-}$ mice is composed of cartilage, chondrocyte maturation is severely inhibited throughout most of the skeleton. *Col2a1*, which is expressed in immature chondrocytes, is expressed in whole *Runx2*$^{-/-}$ skeletons, whereas *Col10a1*, which is expressed in mature chondrocytes, is restrictedly expressed in the tibia, fibula, radius, and ulna. The expression of *Spp1*, *Ibsp*, and *Mmp13*, which are expressed in terminally differentiated chondrocytes, was found to be virtually absent in whole *Runx2*$^{-/-}$ skeletons (Inada et al. 1999). *Spp1* and *Mmp13* are directly regulated by Runx2 (Jimenez et al. 1999; Porte et al. 1999; Sato et al. 1998; Selvamurugan et al. 2000; Hess et al. 2001). These findings indicate that Runx2 is required for chondrocyte maturation (Fig. 6.1). Even in the restricted skeletons of *Runx2*$^{-/-}$ mice, in which chondrocyte maturation occurs, vascular invasion is absent, indicating that Runx2 is also required for vascular invasion into the cartilage (Zelzer et al. 2001; Lee et al. 2012; Himeno et al. 2002). In wild-type mice, osteoblast differentiation occurs in the perichondrium and the bone collar is formed. However, osteoblast differentiation is completely blocked in *Runx2*$^{-/-}$ mice and there are no osteoblasts in the perichondrium (Komori et al. 1997). Therefore, the absence of osteoblasts in the perichondrium may affect chondrocyte maturation. These findings indicate that Runx2 regulates chondrocyte maturation directly or indirectly through the regulation of osteoblast differentiation in the perichondrium.

In the prechondrogenic cell line, ATDC5, the expression of *Runx2* was found to be enhanced prior to differentiation to the hypertrophic phenotype, and a treatment with antisense oligonucleotides for *Runx2* inhibited chondrocyte maturation. The retrovirally forced expression of *Runx2* in chick immature chondrocytes also induced chondrocyte maturation (Enomoto et al. 2000). These findings indicate that Runx2 is an important regulatory factor in chondrocyte maturation (Komori 2000) (Fig. 6.1). The overexpression of *Runx2* in chondrocytes using a *Col2a1* promoter/enhancer has been shown to accelerate chondrocyte maturation and endochondral ossification (Takeda et al. 2001; Ueta et al. 2001). Chondrocyte maturation even occurs in permanent cartilage including articular cartilage, thyroid cartilage, cricoid cartilage, tracheal cartilage, and intervertebral discs, which are replaced with bone in *Runx2* transgenic mice. In contrast, the expression of dn-*Runx2* in chondrocytes decelerates chondrocyte maturation and endochondral ossification (Ueta et al. 2001). Since *Runx2* and dn-*Runx2* are only expressed in chondrocytes, these findings indicate that Runx2 directly regulates chondrocyte maturation (Fig. 6.1).

Tenascin is expressed in chondrocytes once cartilage tissue appears, but becomes limited to articular chondrocytes as cartilage development progresses. The expression of tenascin is absent in the presumptive joint regions of *Runx2* transgenic mice, while it is expressed in most chondrocytes in the skeleton of dn-*Runx2* transgenic mice. These findings indicate that Runx2 inhibits chondrocytes from acquiring the characteristics of permanent cartilage (Ueta et al. 2001) (Fig. 6.1). The mechanisms responsible for the specification of permanent cartilage have not yet been elucidated in detail. In *Runx2* transgenic mice, permanent cartilage undergoes endochondral ossification. Therefore, even permanent cartilage or cartilage fated to be permanent has the potential to be transient cartilage that enters the endochondral pathway. Furthermore, the lack of cell hypertrophy in permanent cartilage appears to be due to negative regulation by microenvironmental cues and mechanisms, which may downregulate the expression of *Runx2*. The degeneration of permanent cartilage is a feature of the pathological changes occurring with osteoarthritis in articular joints. Osteoarthritis is frequently associated with the ectopic expression of a number of molecules such as *Col10a1* (von der Mark et al. 1992; Shlopov et al. 1997), *Spp1* (Pullig et al. 2000), and *Mmp13* (Shlopov et al. 1997), which are normally specific to hypertrophic chondrocytes and are encoded by the direct target genes of Runx2 (Li et al. 2011; Sato et al. 1998; Porte et al. 1999; Hess et al. 2001; Jimenez et al. 1999). Therefore, we previously proposed that the degradation of articular cartilage in osteoarthritis may be related to the uncontrolled behavior of permanent chondrocytes and abnormal expression of the hypertrophic phenotype, and also that Runx2 may be involved in osteoarthritis (Ueta et al. 2001) (Fig. 6.1). In accordance with these hypotheses, the degradation of articular cartilage in *Runx2*$^{+/-}$ mice was previously reported to be significantly reduced in an osteoarthritis mouse model (Kamekura et al. 2006).

Terminally differentiated chondrocytes have been detected in restricted parts of the skeleton in *Runx2*$^{-/-}$ mice, indicating that other transcription factors are also involved in chondrocyte maturation. Runx3 is expressed in prehypertrophic chondrocytes, and chondrocyte maturation is slightly disturbed at E15.5, but not in the newborn stage. Chondrocyte maturation was previously shown to be completely absent in the whole skeleton in *Runx2*$^{-/-}$ *Runx3*$^{-/-}$ mice, indicating that Runx2 and Runx3 have redundant functions in chondrocyte maturation and are essential for chondrocyte maturation (Yoshida et al. 2004).

In *Runx2*$^{-/-}$ mice, the lengths of the limbs are short, chondrocyte proliferation is reduced, and the expression of *Ihh*, which is expressed in prehypertrophic chondrocytes, is severely reduced. Several Runx2-binding motifs have been identified in the promoter region of *Ihh*, and Runx2 directly regulates *Ihh* expression (Yoshida et al. 2004). Therefore, Runx2 regulates not only chondrocyte maturation, but also chondrocyte proliferation through *Ihh* regulation (Komori 2005) (Fig. 6.1).

6.4 Roles of Cbfb in Skeletal Development

Runx1$^{-/-}$ mice and *Cbfb*$^{-/-}$ mice both die at midgestation due to the lack of fetal liver hematopoiesis, indicating that the heterodimerization of Runx1 and Cbfb is required for fetal liver hematopoiesis (Okuda et al. 1996; Sasaki et al. 1996; Wang et al. 1996a, b). Since *Cbfb*$^{-/-}$ mice die between E11.5–13.5, the involvement of Cbfb in skeletal development remains to be clarified. In an attempt to overcome lethality, we and others partially rescued the lack of fetal liver hematopoiesis in *Cbfb*$^{-/-}$ mice, showing the requirement of Cbfb in skeletal development (Kundu et al. 2002; Miller et al. 2002; Yoshida et al. 2002).

In order to precisely evaluate the functions of Cbfb in skeletal development, *Cbfb* was conditionally deleted using *Dermo1* Cre knock-in mice, in which Cre is expressed in mesenchymal cells, giving rise to chondrocyte and osteoblast lineages. The processes of endochondral and intramembranous ossification are both retarded in *Cbfb*$^{fl/fl/Cre}$ mice due to the deceleration of chondrocyte maturation and osteoblast differentiation. Chondrocyte proliferation was also

shown to be reduced in *Cbfb*$^{fl/fl/Cre}$ mice (Qin et al. 2015). Similar findings have been reported in *Cbfb* conditional knockout mice using *Sp7*-Cre mice, *Col2a1* Cre mice, *Prrx1* Cre mice, and *Dermo1* Cre mice (Chen et al. 2014; Fei et al. 2014; Wu et al. 2014a, b; Lim et al. 2015). Although the development of endochondral bones is known to be severely affected in *Cbfb*$^{fl/fl/Cre}$ mice, but not in *Runx2*$^{+/-}$ mice, the development of calvariae and clavicles was affected less in *Cbfb*$^{fl/fl/Cre}$ mice than in *Runx2*$^{+/-}$ mice (Qin et al. 2015). Calvariae and the lateral parts of clavicles are formed through intramembranous ossification (Huang et al. 1997; Marks Jr. and Odgren 2002). Therefore, these findings indicate that Cbfb is vital for chondrocyte maturation and proliferation as well as osteoblast differentiation, and also that Cbfb is crucial for endochondral bone development, but is only partially required for intramembranous bone development.

Runx family protein levels are reduced in *Cbfb*$^{fl/fl/Cre}$ mice, indicating that Cbfb is required for the stability of Runx family proteins (Qin et al. 2015). Cbfb protects Runx2 from polyubiquitination (Lim et al. 2015). However, the levels of reduction differ among Runx family proteins and in cartilaginous limb skeletons and calvariae at E15.5 (Qin et al. 2015). As described above, Runx2 and Runx3 are essential for chondrocyte maturation, and Runx1 is involved in the development of the sternum, occipital bone, and palate by regulating chondrocyte differentiation (Kimura et al. 2010; Liakhovitskaia et al. 2010). Therefore, all Runx family proteins are involved in chondrocyte differentiation. In cartilaginous limb skeletons, the levels of all Runx family proteins are severely reduced in the order of Runx1>Runx3>Runx2, and exist at levels that are 3 %, 8 %, and 13 % those in wild-type mice, respectively. Although the function of Runx1 in the osteoblast lineage is unknown, Runx3 has been shown to play a role in the proliferation of osteoblast lineage cells (Bauer et al. 2015). In calvariae, Runx1 protein levels are the most severely reduced, at 7 % that in wild-type mice, whereas Runx2 and Runx3 protein levels in calvariae are 55 % and 25 %, respectively, those in wild-type mice. Therefore, protein stability differs among Runx family proteins in the absence of Cbfb, with the Runx2 protein being more stable in calvariae than in cartilaginous limb skeletons in the absence of Cbfb. Some unknown proteins may compensate for the lack of Cbfb in calvariae in order to protect against the degradation of the Runx2 protein. The relative stability of the Runx2 protein in the calvariae of *Cbfb*$^{fl/fl/Cre}$ mice explains why *Cbfb*$^{fl/fl/Cre}$ mice show a severe delay in endochondral ossification, but milder deformities in calvariae and the lateral parts of clavicles, which are formed through intramembranous ossification (Qin et al. 2015).

Two functional Cbfb isoforms have been identified: Cbfb1 and Cbfb2. They are formed by alternative splicing using donor splice sites located inside exon 5 and at the 3′ terminus of exon 5, respectively, and an acceptor splice site located at the 5′ terminus of exon 6 (Ogawa et al. 1993). *Cbfb1*$^{-/-}$ mice and *Cbfb2*$^{-/-}$ mice have been generated by mutating donor splice signals (Tachibana et al. 2011). *Cbfb1*$^{-/-}$ mice develop normally, whereas *Cbfb2*$^{-/-}$ mice show dwarfism and endochondral and intramembranous ossification is inhibited (Jiang et al. 2016). Although Cbfb1 and Cbfb2 exhibit similar activities for the stabilization of Runx family proteins, Cbfb1 is more potent at enhancing chondrocyte and osteoblast differentiation and the DNA binding of Runx2. However, the formation of the Cbfb1 isoform is strictly regulated in skeletal tissues, livers, and thymuses, in which Runx family transcription factors play important roles in osteoblast and chondrocyte differentiation, hematopoiesis, and T-cell development, respectively, and *Cbfb1* mRNA levels are one third those of *Cbfb2*. Therefore, Cbfb1 and Cbfb2 have redundant functions with different efficiencies, and modulations in the relative levels of the isoforms appear to adjust transcriptional activation by Runx2 to appropriate physiological levels (Jiang et al. 2016).

6.5 Transcriptional Regulation of the Runx2 Gene

Runx2 is expressed as two isoforms that possess different N-termini (type I Runx2 starting with the sequence MRIPV and type II Runx2 starting with the sequence MASNS), and are expressed under two promoters: the proximal (P2) and distal (P1) promoters, respectively (Fujiwara et al. 1999). Although both isoforms are expressed in osteoblasts and chondrocytes, the expression of *Runx2* in osteoblasts was found to be mainly transcribed from the P1 promoter (Park et al. 2001; Enomoto et al. 2000). The transcriptional regulation of *Runx2* was investigated in the P1 promoter (Fujiwara et al. 1999; Zambotti et al. 2002; Lee et al. 2005; Hassan et al. 2007; Zhang et al. 2009; Gaur et al. 2005), and the findings obtained showed that reporter mice under the control of the P1 promoter failed to express the reporter gene in osteoblasts (Lengner et al. 2002), suggesting the presence of an enhancer for osteoblast-specific expression.

GFP reporter mice using a 200-kb BAC clone of the *Runx2* gene locus, which includes the P1 and P2 promoters, recapitulate the endogenous expression of *Runx2* (Kawane et al. 2014). A 343-bp osteoblast-specific enhancer was identified following the serial deletion of the BAC clone. GFP is specifically expressed in the osteoblasts of GFP reporter mice driven by an enhancer and minimal promoter. The sequence of this enhancer is highly conserved among the mouse, human, dog, horse, opossum, and chicken. It is also highly enriched for histone H3 mono- and dimethylated at Lys4 and acetylated at Lys27 and Lys18, but depleted for histone H3 trimethylated at Lys4 in primary osteoblasts. Furthermore, the histone variant H2A.Z is enriched in the enhancer. These are typical chromatin modifications in enhancers. A 89 bp fragment in the 343-bp enhancer still retains the ability to direct the reporter gene to osteoblasts. Dlx5/6 and Mef2 have been shown to directly bind to the homeobox motif and Mef2-binding motif in the 89-bp core sequence, respectively. Dlx5/6 and Mef2 form an enhanceosome with Tcf7, Ctnnb1, Sox5/6, Smad1, and Sp7, which are integrated into the enhanceosome by a protein-protein interaction, and activate the enhancer. Since *Tcf7* and *Sp7* are known to be regulated by Runx2 (Mikasa et al. 2011; Yoshida et al. 2012), these transcription factors are reciprocally regulated in osteoblast differentiation. Although Msx2 and Dlx5 both bind to the homeobox motif in the enhancer, Msx2 inhibits enhancer activity. The binding of Msx2 to the homeobox motif is dominant in the uncommitted mesenchymal cell line, C3H10T1/2, whereas the binding of Dlx5 to it is dominant in osteoblastic MC3T3-E1 cells. Therefore, the switching of binding from Msx2 to Dlx5 in the homeobox motif is important for activation of the enhancer (Kawane et al. 2014). The 343-bp enhancer is useful for the screening of drugs for osteoporosis and bone regeneration by targeting *Runx2*, and is also important as a vector in gene therapy for bone diseases.

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