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PROSPECT

Runx2, A Multifunctional Transcription Factor in Skeletal Development

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Abstract The identification of Runx2 (runt-related protein 2) function has greatly advanced the understanding of skeletal development over the last 5 years. Runx2 is regulated transcriptionally and post-translationally through the activity of many identified factors, although, the physiological significance of each remains to be demonstrated. The interaction of Runx2 with other transcription factors and cofactors has been shown to be important in Runx2-dependent gene regulation. Runx2 plays important roles in multiple steps of skeletal development. Runx2 determines the lineage of osteoblasts from multipotent mesenchymal cells, enhances osteoblast differentiation at an early stage, and inhibits osteoblast differentiation at a late stage. Runx2 plays crucial roles in chondrocyte maturation and in the specification of cartilage phenotypes. Furthermore, Runx2 is involved in vascular invasion into cartilage and osteoclastogenesis. Therefore, the determination of Runx2 function and the investigation of the cascades of Runx2-dependent gene regulation are important in the elucidation of skeletal biology. J. Cell. Biochem. 87: 1–8, 2002.
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Key words: Runx2; transcription factor; osteoblast; chondrocyte; vascular invasion

The skeletons of vertebrates are constructed through intramembranous or endochondrial ossification at specific developmental times and at specific sites. Intramembranous ossification, which is restricted to the cranial vault, some facial bones, and parts of the mandible and clavicle, is performed by osteoblasts that have differentiated from mesenchymal cells. The remainder of the vertebrate skeleton forms as a cartilage template that is replaced with bone tissues through a sequential processes of endochondrial ossification, which includes chondrocyte maturation, vascular invasion into the cartilage, and bone formation by osteoblasts. Runt-related protein 2 (Runx2)/core binding factor α1 (Cbfa1)/polyoma enhancer binding protein 2αA (Pebp2αA) plays crucial roles in the processes of both intramembranous and endochondrial ossification.

Runx2 is a transcription factor that belongs to the Runx family [Komori and Kishimoto, 1998] (Fig. 1). Three Runx genes (Runx1/Cbfa2/Pebp2αB, Runx2/Cbfa1/ Pebp2αA, and Runx3/Cbfa3/Pebp2αC) have been identified. Each of these genes encodes a DNA-binding domain, runt that is homologous with the Drosophila pair-rule gene runt. However, each Runx family protein has an unique function. Runx1 is essential for definitive hematopoiesis [Komori and Kishimoto, 1998], Runx3 is a major growth regulator of gastric epithelial cells [Li et al., 2002], and Runx2 is essential for skeletal development and is described in detail below. Runx2 was originally cloned from mouse fibroblasts, and its expression has been detected in T-cell lines, NIH3T3 cells, thymus, and testis. A Runx binding sequence, PyGPyGGTPy, has been identified in the regulatory regions of many T-cell-specific genes. The DNA-binding sites of Runx have also been identified in the promoter region of the osteocalcin gene. In addition, Runx1 has been shown to bind to the osteocalcin promoter region and to transcriptionally activate the osteocalcin gene. The involvement of Runx2 in skeletal development was demonstrated

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Runx family	main function	related diseases in humans
Runx1/Cbfa2/Pebp2αB	hematopoietic stem cell differentiation	acute myeloid leukemia
Runx2/Cbfa1/Pebp2αA	osteoblast differentiation, chondrocyte maturation	cleidocranial dysplasia
Runx3/Cbfa3/Pebp2αC	growth regulation of gastric epithelial cells	gastric cancer*
cofactor of Runx		
Cbfb/Pebp2β	essential factor for Runx1	acute myeloid leukemia

Fig. 1. Genes of the Runx family. The main functions of Runx genes and a cofactor gene, Cbfb, and related human diseases are shown. *The relation was suggested because approximately half of gastric cancer cells do not express RUNX3, and RUNX3 suppresses tumorigenesis of a cancer cell line, but a mutation of RUNX3 does not [Li et al., 2002].

by Runx2-deficient ($Runx2^{-/-}$) mice, which showed a complete lack of bone formation [Komori and Kishimoto, 1998].

Mice carrying a heterozygous mutation in the Runx2 locus showed a phenotype similar to cleidocranial dysplasia in humans, which is an autosomal-dominant disease characterized by hypoplastic clavicles, open fontanelles, supernumerary teeth, and short stature [Komori et al., 1997; Otto et al., 1997]. Further, mutations of the Runx2 gene have been found in patients with cleidocranial dysplasia [Lee et al., 1997; Mundlos et al., 1997], indicating that a heterozygous mutation in the Runx2 locus results in cleidocranial dysplasia (Fig. 1).

REGULATION OF RUNX2

Runx2 is expressed as two isoforms (type I Runx2 starting with the sequence MRIPV and type II Runx2 starting with the sequence MASNS) that possess different N-termini, and are expressed under different promoters [Komori and Kishimoto, 1998]. Both type I and II Runx2 isoforms are expressed in chondrocytes, as well as osteoblasts, although, type II Runx2 expression is predominant in osteoblasts [Enomoto et al., 2000; Banerjee et al., 2001]. The two isoforms have been observed to be functionally similar in chondrocytes [Ueta et al., 2001]. Runx2 is transcriptionally upregulated by bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), and retinoic acid (RA), and is downregulated by $1,25(OH)_2D_3$ and tumor necrosis factor-α (TNF-α) [Ducy et al., 1997; D'Souza et al., 1999; Jiménez et al., 2001; Drissi et al., 2002; Gilbert et al., 2002] (Fig. 2). The transcriptional regulation of Runx2 by transforming growth factor-β (TGF-β) seems to depend on the specific cell types used. TGF-β upregulates Runx2 expression in C2C12 cells [Lee et al., 2000], but downregulates Runx2 expression in primary osteoblasts and ROS17/2.8 cells [Alliston et al., 2001]. However, the factors that are involved in the control of Runx2 expression at specific times and locations during the differentiation of osteoblasts and chondrocytes remains to be clarified.

The regulatory region of Runx2 has multiple Runx2 binding sites, and Runx2 has been shown to regulate the activity of its own promoter both positively [Ducy et al., 1999] or negatively [Drissi et al., 2000]. Our experiments using Runx2 and dominant-negative (DN) Runx2 transgenic mice showed no obvious autoregulation of Runx2 [Liu et al., 2001; Ueta et al., 2001]. However, our recent experiments suggest that Runx2 negatively regulates its own promoter in osteoblast precursors (submitted). The negative feedback of Runx2 expression may be an important regulatory step in the onset of osteoblast differentiation. Post-translational

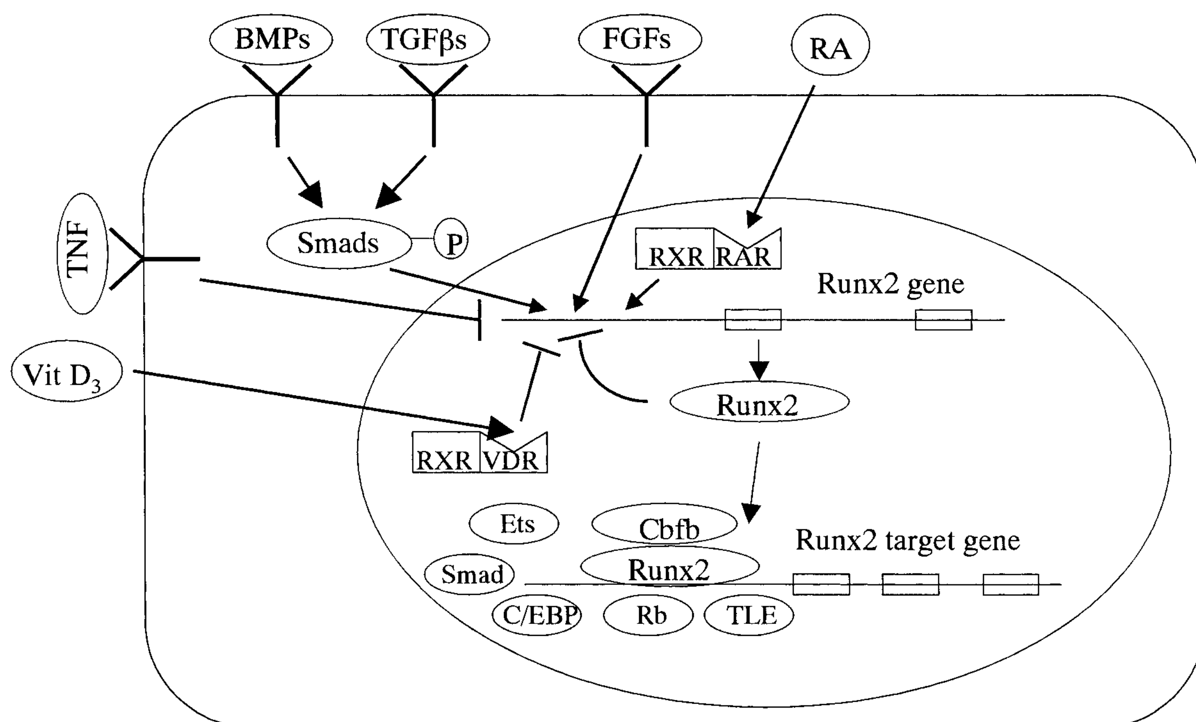

Fig. 2. The regulation of Runx2. Runx2 transcription is regulated positively by BMPs, FGFs, and RA, and negatively by TNF-α, 1,25$(OH)_2D_3$ (Vit D_3), and Runx2 itself. Both positive and negative regulation of Runx2 by TGF-βs have been reported. Runx2 forms a heterodimer with Cbfb and interacts with many transcription factors and cofactors during Runx2-dependent transcriptional regulation.

regulation of Runx2 has also been reported. MAPK-dependent phosphorylation of Runx2 stimulates Runx2-dependent transcription [Xiao et al., 2000]. Protein kinase A also phosphorylates the transactivation domain of Runx2 during the process of matrix metalloproteinase 13 (MMP13) activation by parathyroid hormone (PTH) [Selvamurugan et al., 2000]. The cAMP pathway, a major intracellular pathway mediating PTH signals, suppresses Runx2 through proteolytic degradation that involves a ubiquitin/proteasome-dependent mechanism [Tintut et al., 1999].

Runx2 interacts with other transcription factors, such as Ets, Smad, and C/EBP, with the transcriptional cofactor Rb, and with the transcriptional repressor TLE. These interactions greatly influence Runx2 function [Sato et al., 1998; Javed et al., 2000; McCarthy et al., 2000; Zhang et al., 2000; Thomas et al., 2001; Gutierrez et al., 2002] (Fig. 2). Further, the fidelity of the subnuclear localization of Runx2 mediated by a nuclear-matrix-targeting signal is required for Runx2 function [Choi et al., 2001]. The transcriptional cofactor, core binding factor-β (Cbfb), which does not have DNA binding capacity, forms a heterodimer with the runt protein, a DNA-binding domain that is common to the Runx family, and enhances the in vitro DNA binding of the runt protein [Tahirov et al., 2001]. Cbfb$^{-/-}$ mice die at the mid-gestational stage owing to the lack of definitive hematopoiesis, a phenotype similar to Runx1$^{-/-}$ mice, indicating that Cbfb is essential for the function of Runx1 in vivo [Komori and Kishimoto, 1998]. However, the necessity of Cbfb for the function of Runx2 has been controversial. Cbfb enhanced the promoter activities of osteocalcin and osteopontin induced by Runx2 [Harada et al., 1999], but Runx2 failed to form a heterodimer with Cbfb in vitro [Thirunavukkarasu et al., 1998]. Our recent work has demonstrated that Cbfb plays crucial roles in Runx2-dependent skeletal development by enhancing the DNA binding capacity of Runx2 (submitted).

RUNX2 AND OSTEOBLAST DIFFERENTIATION

Runx2$^{-/-}$ mice die just after birth, due to a failure to breath [Komori et al., 1997; Otto et al., 1997]. These mice completely lack both endochondrial and intramembranous ossification

due to the absence of osteoblasts, demonstrating that Runx2 is an essential factor for osteoblast differentiation. Further, Runx2$^{-/-}$ calvarial cells, which completely lacked the ability to differentiate into osteoblasts, retained the ability to differentiate into adipocytes and chondrocytes [Kobayashi et al., 2000]. Taken together, these findings suggest that Runx2 plays an essential role in steering multipotent mesenchymal precursor cells toward an osteoblastic lineage [Komori, 2000] (Fig. 3). Further, mesenchymal condensations in the regions of future membranous bones were observed in Osterix$^{-/-}$ mice, but not in Runx2$^{-/-}$ mice [Komori et al., 1997; Nakashima et al., 2002], suggesting that Runx2 is essential for mesenchymal condensation, which is an early step in skeletogenesis.

Runx2 transgenic mice under the control of the type I collagen promoter indicate that Runx2 promotes osteoblast differentiation at an early stage, but inhibits osteoblast differentiation at a late stage. The expression of osteoblastic markers, including α1(I) collagen, alkaline phosphatase, MMP13, and osteocalcin, which are upregulated according to the osteoblast maturation, are decreased in the transgenic mice irrespective of the increase of the number of osteoblasts [Liu et al., 2001] (Fig. 3). In these mice, most of osteoblasts exhibit a less mature phenotype, and the numbers of terminally differentiated osteoblasts, which strongly express osteocalcin, and osteocytes were diminished greatly. This finding is surprising because a large number of recent in vitro studies suggest that Runx2 is a positive regulator that can upregulate the expression of bone matrix genes, including type I collagen, osteopontin, bone sialoprotein (BSP), osteocalcin, and fibronectin. [Ducy et al., 1997; Harada et al., 1999; Lee et al., 2000]. Runx2-dependent transcriptional activation has also been shown to encompass many promoters, including α1(I) collagen, α2(I) collagen, osteopontin, osteocalcin, MMP13, and osteoprotegerin (OPG) [Harada et al., 1999; Jiménez et al., 1999; Thirunavukkarasu et al., 2000; Kern et al., 2001]. However, the BSP promoter is an exception, since Runx2 represses its activity [Javed et al., 2001]. Further, the overexpression of DN-Runx2 at a late stage of osteoblast differentiation results in osteopenia, with a decreased expression of the genes, encoding main bone matrix proteins, including α1(I), α2(I) collagen, osteopontin, BSP, and osteocalcin [Ducy et al., 1999]. These findings suggest that the presence of cofactors or other transcription factors, which is dependent on the maturational stage of osteoblastic cells, greatly influences Runx2-dependent gene regulation in vivo. Therefore, the suppressed expression of the late osteoblastic markers in immature osteoblasts may be due

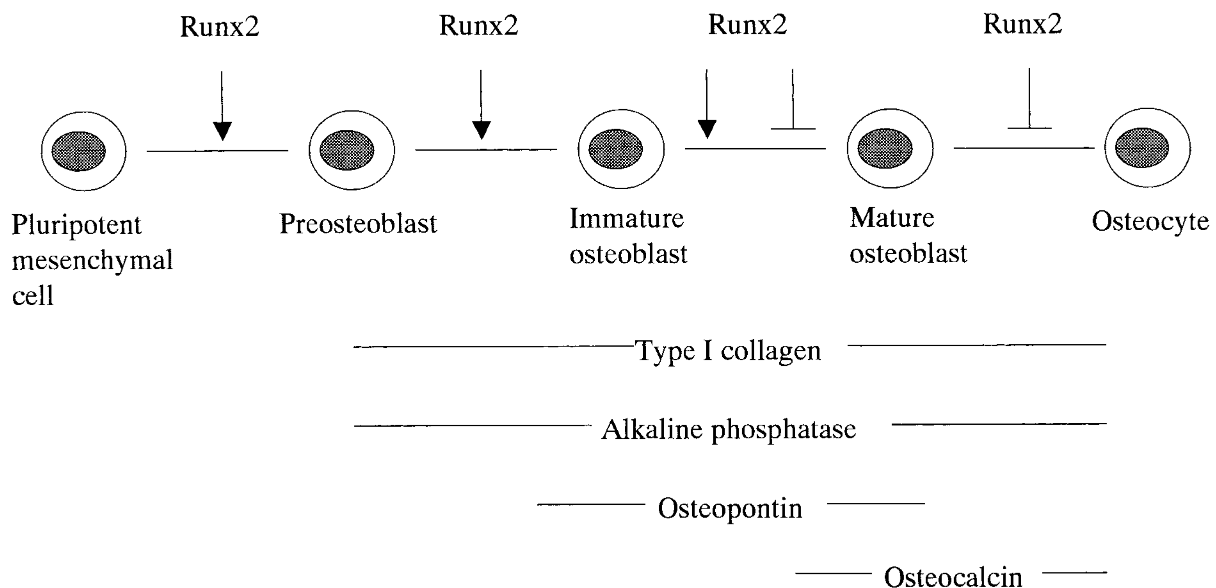

Fig. 3. Role of Runx2 in osteoblast differentiation. Runx2 determines the osteoblast lineage from pluripotent mesenchymal cells, enhances osteoblast differentiation at an early stage, and inhibits osteoblast differentiation at a late stage. The expression patterns of osteoblastic markers are also shown.

to either the interaction of Runx2 with transcriptional suppressors, such as TLE or insufficient expression of transcriptional activators that interact with Runx2. However, the contribution of Runx2 to the production of bone matrix proteins in vivo needs to be re-examined.

RUNX2 AND CHONDROCYTE DIFFERENTIATION

In Runx2$^{-/-}$ mice, whose entire skeleton is composed of cartilage, chondrocyte differentiation is severely disturbed throughout most of the skeleton, and no vascular invasion is observed [Inada et al., 1999; Kim et al., 1999]. All Runx genes are expressed in chondrocytes, and Runx2 expression is detected in mesenchymal condensations [Inada et al., 1999; Kim et al., 1999; Levanon et al., 2001; Stricker et al., 2002]. Since, DN-Runx2, which inhibits all Runx proteins, inhibits the cellular condensation of a prechondrogenic cell line ATDC5, Runx proteins may play a role in an early step of chondrogenesis [Akiyama et al., 1999]. However, Runx2$^{-/-}$ mice develop a cartilaginous skeleton [Komori et al., 1997; Otto et al., 1997], and the introduction of a Runx2-containing retrovirus into the chick limb bud fails to induce ectopic cartilage [Stricker et al., 2002]. Therefore, the involvement of Runx proteins in the formation of cartilaginous anlagen should be examined (Fig. 4). In ATDC5 cells, Runx2 expression is elevated prior to differentiation to the hypertrophic phenotype, and treatment with antisense oligonucleotides for Runx2 inhibit chondrocyte maturation. Further, retrovirally forced expression of Runx2 in chick immature chondrocytes induces chondrocyte maturation [Enomoto et al., 2000]. These results indicate that Runx2 is an important regulatory factor in chondrocyte maturation [Komori, 2000] (Fig. 4).

These findings were confirmed by Runx2 transgenic mice under the control of the type II collagen promoter [Takeda et al., 2001;

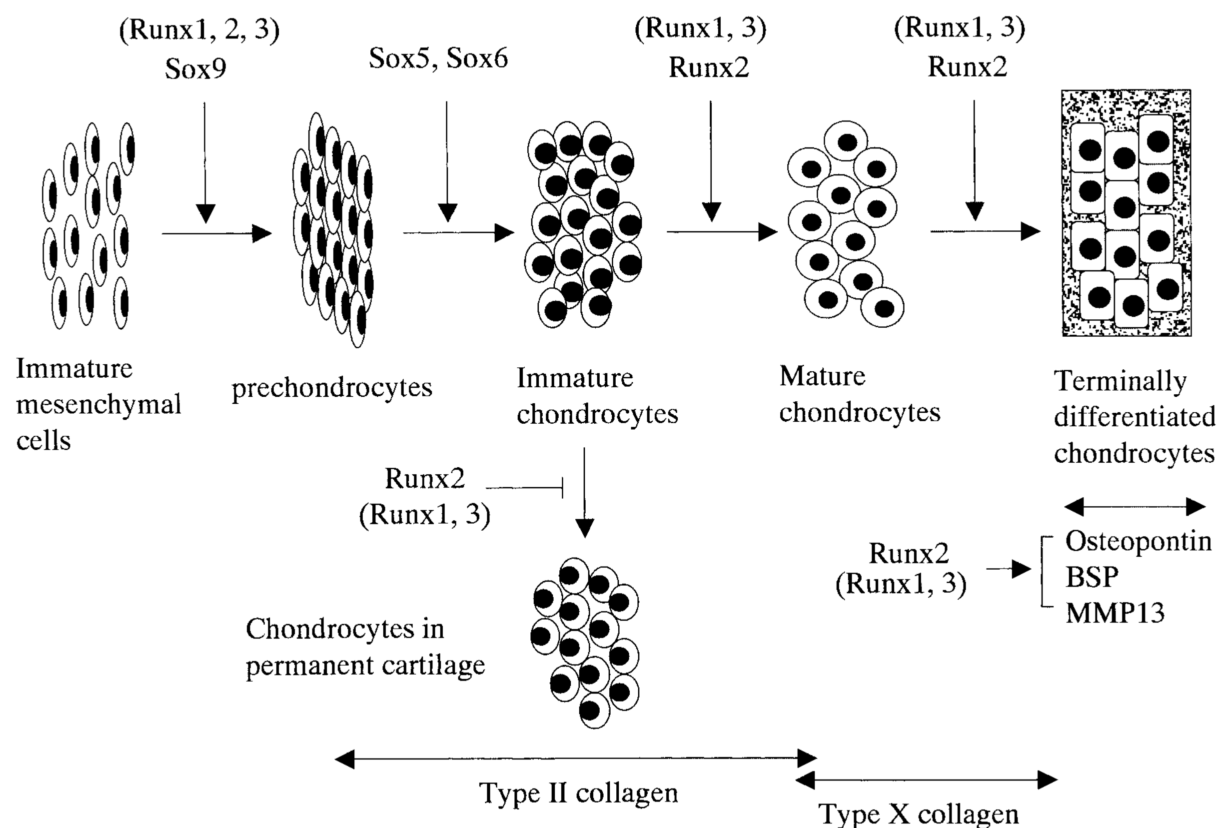

Fig. 4. Role of Runx2 in chondrocyte differentiation. Runx2 is required for chondrocyte maturation, and controls the expression of osteopontin, BSP, and MMP13 in terminally differentiated chondrocytes. Runx2 inhibits chondrocytes from acquiring the phenotype of permanent cartilage. Runx1 and Runx3 also seem to contribute to these processes. Runx proteins may be involved in the process of mesenchymal condensation at an early step. Sox9, which is essential for mesenchymal condensation, and Sox5 and Sox6, which are required for high levels of expression of cartilage matrix genes, are also shown.

Ueta et al., 2001]. In these mice, chondrocyte maturation and endochondrial ossification are greatly accelerated. Further, the introduction of the transgene into Runx2$^{-/-}$ mice rescues chondrocyte maturation in Runx2$^{-/-}$ mice [Takeda et al., 2001]. In contrast, endochondrial ossification is completely blocked, and the cartilages are composed of immature chondrocytes in DN-Runx2 transgenic mice, demonstrating that Runx2 is a fundamental transcription factor for chondrocyte maturation [Ueta et al., 2001]. Since, the inhibition of chondrocyte maturation in DN-Runx2 transgenic mice is more severe than that in Runx2$^{-/-}$ mice, other Runx proteins may also be involved in chondrocyte maturation. Furthermore, the results of these experiments reveal another role of Runx2 in chondrogenesis. Runx2 transgenic mice fail to form most of their joints, and the permanent cartilage entered into the process of endochondrial ossification, whereas most of the chondrocytes in DN-Runx2 transgenic mice retain the phenotype of permanent cartilage [Ueta et al., 2001]. Joint fusions are also observed in chick limbs infected with Runx2 containing retrovirus [Stricker et al., 2002]. These findings demonstrate that Runx2 plays an important role in the specification of cartilage phenotype (Fig. 4). The mechanisms used to suppress Runx2 expression in permanent cartilage need to be examined, because they may be involved in the pathogenesis of degenerative diseases of permanent cartilage, such as osteoarthritis.

VASCULAR INVASION OF CARTILAGE AND OSTEOCLASTOGENESIS

Runx2$^{-/-}$ mice completely lack vascular invasion into cartilage, irrespective of the presence of calcified cartilage in some regions of the skeleton, including the tibia, fibula, radius, and ulna. These findings suggest that Runx2 is involved in vascular invasion [Inada et al., 1999; Kim et al., 1999]. However, the function of Runx2 in vascular invasion is unclear. Although, vascular endothelial growth factor (VEGF) is a candidate for the lack of vascular invasion in Runx2$^{-/-}$ mice, VEGF expression in Runx2$^{-/-}$ cartilage is controversial [Zelzer et al., 2001; Himeno et al., 2002]. After vascular invasion occurs, the hematopoietic system, including endothelial cells, affects growth plate vascularization. When Runx2$^{-/-}$ cartilage is transplanted into the spleen of wild-type mice, vascular invasion into the cartilage occurs, but is severely retarded compared with wild-type cartilage, suggesting that Runx2-dependent gene regulation in terminally differentiated chondrocytes is important for vascular invasion [Himeno et al., 2002]. Osteopontin, BSP, and MMP13, which are severely reduced in Runx2$^{-/-}$ terminally differentiated chondrocytes, are involved in vascular invasion into cartilage through the enhancement of osteoclast attachment and the cleavage of type II collagen (Fig. 4). However, additional Runx2-dependent gene regulation in terminally differentiated chondrocytes is required for efficient vascular invasion [Himeno et al., 2002]. These Runx2 target genes that are important for proper vascular invasion remain to be identified.

A lack of osteoclasts in Runx2$^{-/-}$ mice suggests a potential role of Runx2 in osteoclastogenesis [Komori et al., 1997]. Receptor activator of NF-κB ligand (RANKL)-RANK signaling is essential for osteoclastogenesis, and OPG, which is a decoy receptor of RANKL, inhibits RANKL-RANK signaling. The 5′ flanking region of the RANKL gene has two putative Runx binding sites, and RANKL expression is severely decreased in Runx2$^{-/-}$ mice [Gao et al., 1998; Kitazawa et al., 1999]. Although, Runx2 was able to bind to these sites, no transcriptional activation is observed [O'Brien et al., 2002]. In contrast, the 5′ flanking region of OPG has many putative Runx binding sites, and Runx2 stimulates OPG promoter activity [Thirunavukkarasu et al., 2000]. However, in Runx2 transgenic mice, with Runx2 under the control of the type I collagen promoter, both RANKL and OPG expression are decreased in adult mice [Liu et al., 2001]. Further, our recent data demonstrate that Runx2 induces RANKL expression and inhibits OPG expression in immature mesenchymal cells (unpublished communications). Although, the regulation of RANKL and OPG expression by Runx2 seems to be dependent on the maturational stage of osteoblast lineage cells, the role of Runx2 in osteoclastogenesis needs to be further investigated.

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