EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 34, No. 6, 426-433, December 2002

Spatio-temporal expression patterns of Runx2 isoforms in early skeletogenesis

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Accepted 25 November 2002

Abbreviations: Col-II, collagen type II; Col-X, collagen type X; PBS, phosphate-buffered saline; PFA, paraformaldehyde

Abstract

Skeletogenesis occurs through either intramembranous or endochondral ossification. In addition, some parts of the skeletal components maintain their cartilaginous characteristics throughout life without mineralization. Runx2 is known to be a pivotal transcription factor for all skeletogenic processes. In this study, we examined the expression patterns of two major isoforms of Runx2 in early skeletogenesis. During intramembranous bone formation, Runx2-type I (Runx2-I) was widely expressed in osteoprogenitor cells and active osteoblasts, while Runx2-type II (Runx2-II) expression was stringently restricted to cells lining mineralized bones. Cells in permanent cartilage expressed collagen type II (Col-II) but never expressed Runx2 or Col-X. These permanent cartilages were well circumscribed by Runx2-I positive cells, in which Runx2-II was negative. In endochondral bone formation, Runx2 expression temporarily disappeared in Col-II-positive proliferating chondrocytes, but a secondary surge of Runx2-I expression occurred in the prehypertrophic zone before the mineralization of cartilage. Collectively, both Runx2 isoforms showed very similar expression patterns in active bone forming areas; however, Runx2-I has an exclusive role in the early commitment stage of intramembranous or endochondral bone forming processes or in cells surrounding permanent cartilage.

Keywords: chondrocytes; chondrogenesis; collagen type-II; osteoblasts; osteogenesis; transcription factors

Introduction

The craniofacial area is anatomically a very complex part of the body since it is comprised of many bones and cartilages originating from two different sources, the paraxial mesoderm and the cranial neural crest. Though it is widely accepted that facial bones originate from neural crest cells, the origin of flat bones in the calvaria remains controversial. Apart from its cellular origins, craniofacial skeletogenesis occurs through three distinct processes: intramembranous bone formation, endochondral bone formation, and permanent cartilage formation. Flat bones in the calvarium and facial bones undergo typical intramembranous bone formation in which undifferentiated mesenchymal cells transform directly into osteoblasts. On the other hand, long bones in the extremities, axial skeletons and some bones in the cranial base develop through endochondral bone formation. In the meantime, some structures such as the nasal septum and ear cartilages maintain their cartilaginous characteristics throughout life.

The runt-related gene 2 (Runx2, previously known as Cbfa1/Pebp2aA/AML3) is a sequence-specific DNA binding transcription factor that recognizes 5'-PuACC-PuCA-3' or its complementary sequence 5'-TGPyG-GTPy-3' (Ogawa *et al.*, 1992; Melnikova *et al.*, 1993). Homozygous disruption of the gene completely blocks mineralization of all skeletal elements (Komori *et al.*, 1997). This result suggests that Runx2 plays a crucial role in bone development. The requirement of Runx2 for intramembranous bone formation was established by the heterozygous disruption of the gene, which resulted in a cleidocranial dysplasia characterized by hypoplastic clavicles and delayed cranial suture closure because both sites underwent typical intramembranous bone formation (Mundlos *et al.*, 1997; Otto *et al.*, 1997). Meanwhile, an involvement of the gene in endochondral bone formation has been demonstrated by the fact that type II collagen promoter-driven Runx2 expression resulted in the complete mineralization of cartilages in the animal (Takeda *et*

al., 2001; Ueta *et al.*, 2001). These results imply that Runx2 is an indispensable transcription factor for both types of osteogenesis.

Intensive investigations of Runt-related genes structures and the expression of all Runx genes (Runx1, Runx2, and Runx3) showed that it is controlled by two distinct promoters (Stewart *et al.*, 1997; Bangsow *et al.*, 2001; Levanon *et al.*, 2001). Since all Runx proteins bind to a similar DNA consensus sequence (Melnikova *et al.*, 1993; Ogawa *et al.*, 1993), their temporal and spatial expression during development is probably tightly regulated. We previously showed that two major isoforms of Runx2 demonstrated distinct expression patterns during mouse calvarial development (Park *et al.*, 2001). Runx2-type I (Runx2-I, the proximal promoter product) expression is widespread in under-differentiated sutural mesenchymal cells, in pre-osteoblasts in osteogenic fronts, in osteopontin- and osteocalcin-positive osteoblasts lining parietal bones, and in cells surrounding primordial cartilage under the developing calvaria. These results indicate that Runx2-I plays an important role in several steps of cellular differentiation in osteoblasts. In the intramembranous bone formation, Runx2-I is probably involved in an initial commitment step and continues to exert its effects until the final step of the differentiation process. Since the expression of Runx2-type II (Runx2-II, the distal promoter product) is restricted to later events of cell differentiation, being found only in pre-osteoblasts and osteoblasts, its contribution seems to be in the final step of osteoblast differentiation. However, until recently, no studies have examined the expression pattern of the two isoforms of Runx2 in other types of skeletogenesis, such as endochondral bone formation or permanent cartilage formation. In this paper, the spatio-temporal expression patterns of two major Runx2 isoforms in early skeletal development were analyzed by *in situ* hybridization.

Materials and Methods

Preparation of probes

To assess the expression patterns of the Runx2 isoforms, we used two different mouse Runx2 complementary DNA constructs as previously described (Park *et al.*, 2001). Briefly, pRunx2-type I (Runx2-I), a 556-base pair (bp) type I-specific sequence from the 5'-UTR of mouse Runx2-I, was digested with *Xba*I and *BsaWI*, and then subcloned into the *Xba*I site of the pBluscript vector. pRunx2-type II (Runx2-II) is specific for transcripts regulated by the distal promoter and was subcloned from the 5'-UTR and a small part of the N-terminal coding sequence of Runx2-II using *Bam*HI (412 bp). The orientation of each cloned cDNA construct was checked by restriction digestion and sequencing. Collagen type II (Col-II) and collagen type X (Col-X) probes were prepared as described (Inada *et al.*, 1999).

Tissue preparation

Heads of ICR mice aged embryonic day 12.5 (E12.5), E15.5 and E18.5 were fixed overnight at 4°C in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). The mouse heads were dehydrated by a series of ethanol steps and embedded in paraffin. Thin (5 μm) coronal sections passing through the center of the eyes and maxillary molar tooth buds (Figure 1) were mounted on silanized slides, dried overnight at 37°C, and stored at 4°C. Sections were

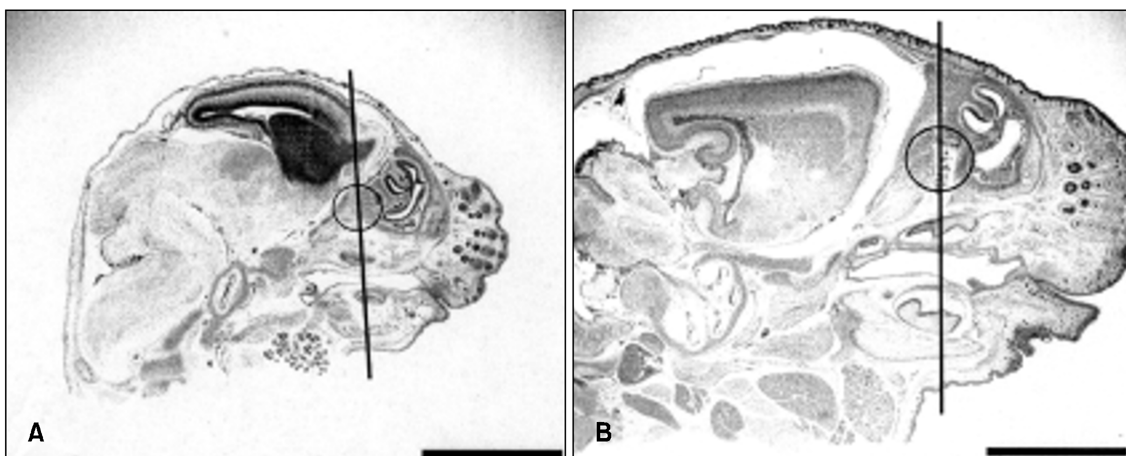

Figure 1. The plane of the coronal sections used for the investigation of craniofacial structures. The section passed through the center of the eyes and maxillary molar tooth buds (black line). The black circle indicates the position of the eyes. A, E15.5; B, E18.5; bar, 200 μm.

stained with hematoxylin and eosin or alcian blue to assess the histology of the developing tissues.

Caudal vertebrae of ICR mice aged embryonic day 16.5 (E16.5), postnatal day 1 (P1) and P5 were fixed overnight at 4°C in 4% PFA in PBS. Postnatal samples were decalcified at 4°C in 12.5% EDTA/2.5% PFA in PBS for about 10 days, with a solution change every 4th day. The tail vertebrae were dehydrated by a series of ethanol steps and embedded in paraffin. Five-micrometer sections passing through the center of the vertebral axis and including coccygeal vertebra number 4 to 6 were mounted on silanized slides for histological staining or *in situ* hybridization.

In situ hybridization

The pRunx2-I gene was digested with *Sac*I or *Hin*dIII and antisense, and sense riboprobes were produced by T3 and T7 RNA polymerase, respectively. The pRunx2-II was digested with *Xho*I or *Sac*I, and antisense and sense riboprobes were produced by T3 and T7 RNA polymerase, respectively. Col II and Col X probes were prepared as previously described (Inada *et al.*, 1999). In situ hybridization on tissue sections was performed using [^{35}S]UTP-labeled riboprobes as described previously (Park *et al.*, 2001). For [^{35}S]UTP-labeled riboprobes, final probe concentrations were adjusted to between 50,000 and 60,000 cpm/μl. After 2 min of denaturation at 80°C, 20-100 μl of probe solution was placed on each slide and covered in parafilm. After overnight hybridization in a humidified sealed box at 52°C, high stringency washes with 50% formamide and 20 mM dithiothreitol (DTT) at 65°C were carried out. Slides were then prepared for autoradiography. The dehydrated slides were dipped into photographic emulsion (Kodak NTB-2; Eastman Kodak, Rochester, NY), dried, and exposed for between 2 and 3 weeks at 4°C. The slides were then developed (Kodak D-19; Eastman Kodak), fixed (Kodak Unifix; Eastman Kodak), and then briefly counterstained with hematoxylin or methyl green and mounted with DePeX (BDG). The slides were examined with the fluorescence microscope; the tissue morphology was examined under the bright field and the location of the radiolabeled probe hybridization was examined under the dark field. The dark field image was transformed to red color and overlapped with the bight field image with Adobe Photoshop.

Results

The craniofacial bone formation begins from embryonic day 12.5 (E12.5). The primordium of future cartilage was weakly stained by alcian blue (Figure 2A) and similarly hybridized with type II collagen (Col II, Figure 2D) in the cartilaginous cranium (CC) of the cranial base. Though intramembranous bone formation of parietal bones had not yet started, Runx2-I was definitely expressed in mesenchymal cells of future calvarial bones and parts of the maxilla and mandible at this stage (Figure 2B). Runx2-II expression was only determined in the primary ossification center near the temporal bones and parts of the maxilla and mandible but was never determined in non-ossified sutural mesenchyme covering the cranial vault (Figure 2C). It is noteworthy that Col II expression was determined in the whole calvaria covering the cranial vault (Figure 2D, arrow), maxilla and mandible (Figure 2I, arrow), in which characteristic chondrocytes did not exist.

With further development of craniofacial structures (E15.5 and E18.5), alcian blue staining (Figure 2, F and K) and Col II hybridization (Figure 2, I and N) were still co-localized in the cartilaginous cranium (CC), nasal cartilage (NC), nasal septal cartilage (SC), and Meckel's cartilage (MC). Runx2-I was strongly expressed in the bony cranium (BC), the maxilla, and the mandible; in other words, it was expressed in all areas of intramembranous bone formation (Figure 2, G and L). Runx2-I was also strongly expressed in the perichondrium circumscribing Col II positive cartilaginous tissues, such as cartilaginous cranium, nasal septal cartilage and Meckel's cartilage. However, the Runx2-I-positive area (Figure 2, G, L and Q) did not overlap with Col II positive chondrocytes (Figure 2, I, N and S) at these stages. In addition, Runx2-I was still strongly expressed in the sagittal suture area between developing parietal bones, in which poorly differentiated mesenchymal cells reside. The expression of Runx2-II was similar to that of Runx2-I, but it was not detected in the sutural mesenchyme between two parietal bones. Moreover, Runx2-II expression did not demarcate around Col II positive cartilages as Runx2-I did (Figure 2. H, M and R). Col X expression was never detected in these craniofacial structures until E18.5 (Figure 2, E, J, O and T). Sense probes did not show any significant hybridization with these tissues (data not shown).

Significantly, Runx2 isoforms were differentially expressed around developing cartilages. Runx2-I positive cells circumscribed Col II positive cartilages in the chondrocranium, nasal cartilage and Meckel's cartilage, while Runx2-II positive cells were not prominent around the permanent cartilage. As these cartilages were surrounded by bones undergoing intramembranous bone formation, the difference in the expression of both isoforms was not very prominent. However, in the case of nasal septal cartilage, the difference was much more clearly demonstrated. Runx2-I was clearly expressed in the cell layer of the

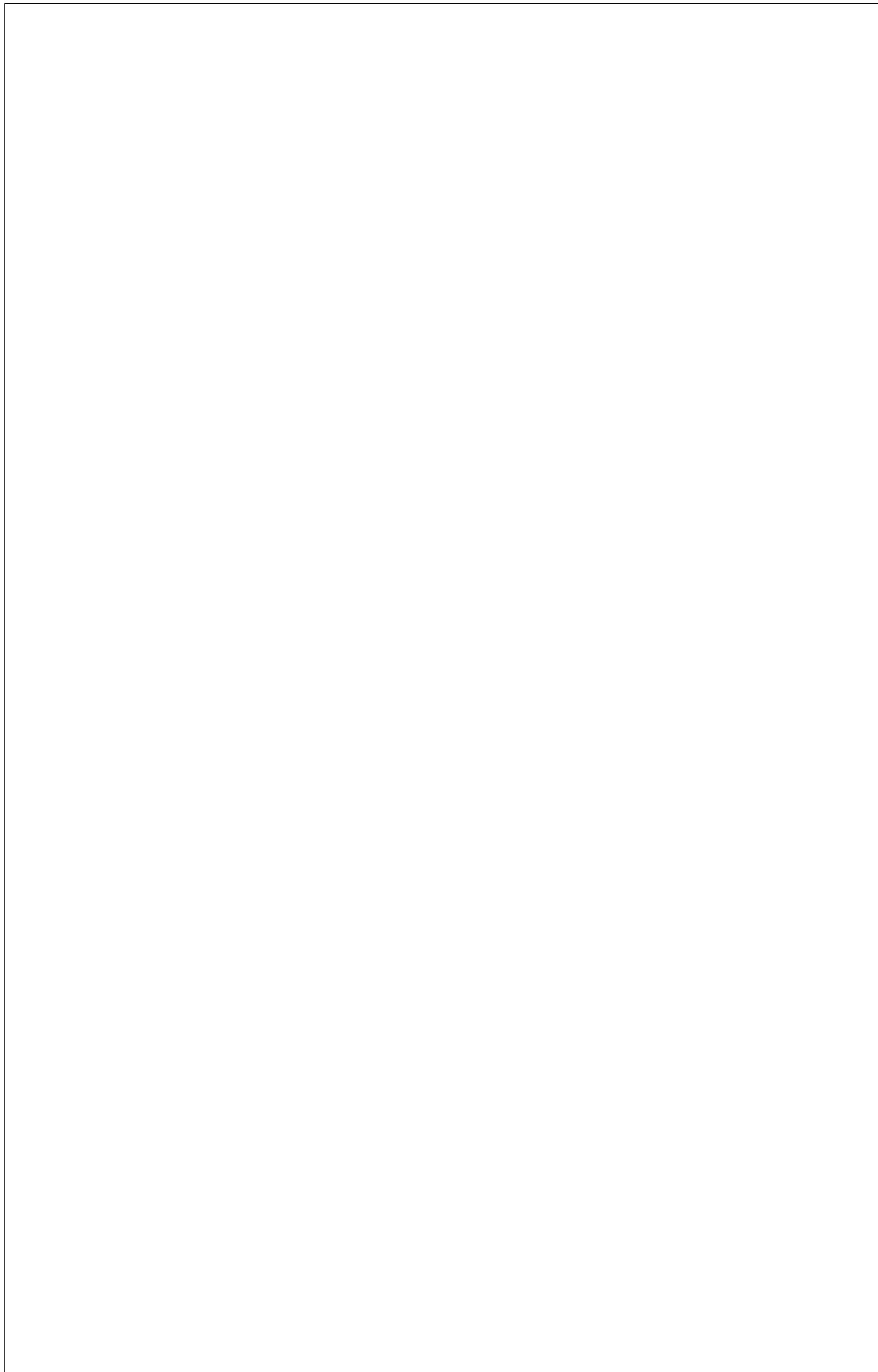

Figure 2. Expression pattern of Runx2 isoforms, Runx2-I and Runx2-II, and chondrocyte marker genes, type II (Col II) and type X collagen (Col X) in a developing embryonic mouse head. Alcian blue staining (A, F, K and P) and *in situ* hybridization of Runx2-I (B, G, L and Q), Runx2-II (C, H, M and R), Col II (D, I, N and S), and Col X (E, J, O and T) were performed with developing mouse heads at embryonic day 12.5 (E12.5; A-E), E15.5 (F-J) and E18.5 (K-O) and nasal septum at day E15.5 (P-T). The developing cranium consists of bony cranium (BC), cartilaginous cranium (CC), and mesenchymal cranium (MC). LV, lateral ventricle; MC, Meckel's cartilage; Mn, mandible; Mx, maxilla; NC, nasal cartilage; P, palate; PB, palatine bone between palatine groove; V, primordium of vomers; SC, nasal septal cartilage; SM, sutural mesenchyme; T, tongue; TF, tooth follicle; VC, vomeronasal cartilage; VO, vomeronasal organ; asterisk, primordium of nasal septum; arrowhead, cellular condensation of perichondrium; arrow, Col II expression in intramembranous bone formation area. Bar indicates 400 μm.

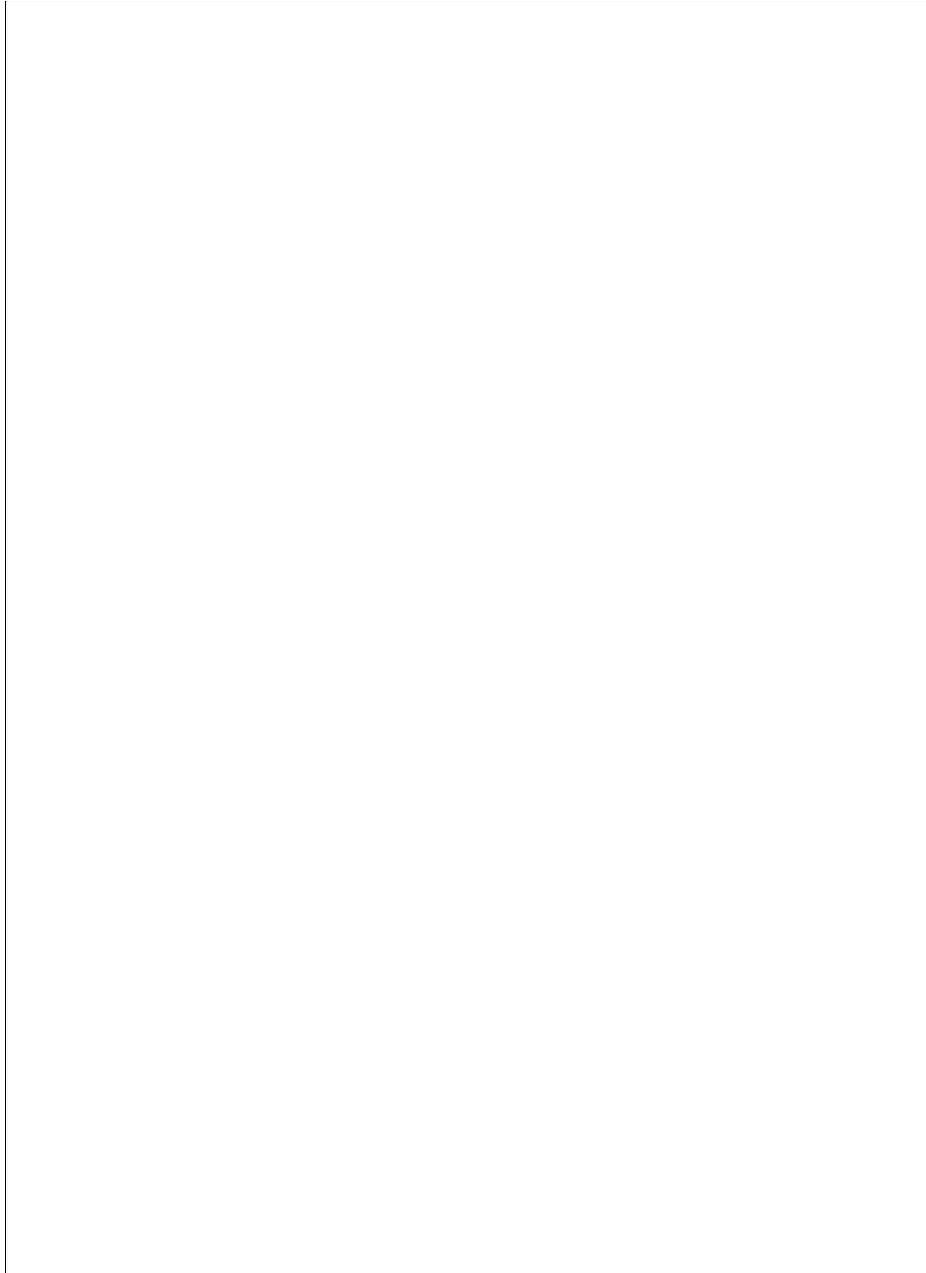

Figure 3. Expression pattern of Runx2-I, Runx2-II, Col-II, and Col-X in tail vertebrae of a developing mouse. Alcian blue staining (A, F, and K) and in situ hybridization of Runx2-I (B, G, and L), Runx2-II (C, H and M), Col-II (D, I, and N) and Col-X (E, J and O) were performed with mouse-tail, including coccygeal vertebra number 4, 5 and 6 at embryonic day 16.5 (E16.5; A-E), postnatal day 1 (P1; F-J) and day 5 (P5; K-O). C, calcified zone; ID, primordium of intervertebral disk; H, hypertrophic zone; asterisk, prehypertrophic and early hypertrophic chondrocytes. Bar indicates 200 μm.

perichondrium, while Runx2-II was not expressed in this area.

Developing mouse caudal vertebrae are believed to be a good model of endochondral bone formation (Marks and Hermey, 1997). The tissue sections were stained with alcian blue (Figure 3A, F and K). Col-II was expressed strongly in the alcian blue stained areas (Figure 3D, I and N). During the earlystage of vertebral development, both isoforms of Runx2 hybridized to the cellular condensation of the perichondrium and to the central part of each segment, where proliferating chondroprogenitor cells resided (Figure 3B and C). At later stages of endochondral bone development, Runx2 expression disappeared in

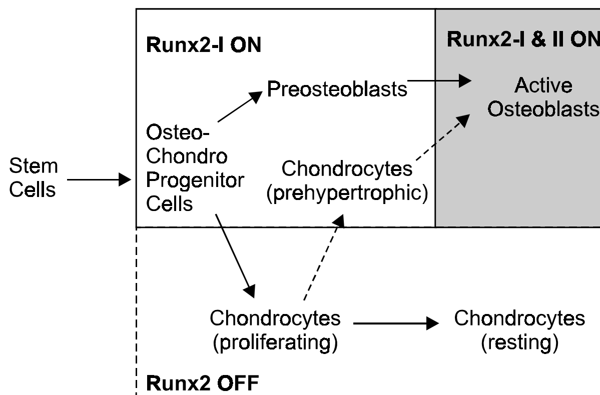

Figure 4. Schematic depiction of differential expression of Runx2 isoforms in skeletogenesis. Thick solid line, intramembranous bone formation; the dotted line, endochondral bone formation; the thin solid line, permanent cartilage formation.

Col-II positive chondrocytes in the proliferating zone. With the maturation of chondrocytes, a secondary surge of Runx2-I expression occurred in the prehypertrophic and early hypertrophic chondrocytes (asterisks, Figure 3G and L) around the Col X positive hypertrophic zone (Figure 3J and O). Runx2- II was not expressed in the early hypertrophic chondrocytes. Neither Runx2 isoform was expressed in hypertrophic chondrocytes, but they were commonly expressed in calcified trabecular bone at the center of each vertebra (Figure 3F and J). Sense probes did not show any significant hybridization with these tissues (data not shown).

Discussion

Mouse skeletal development begins with mesenchymal condensation, which is identifiable at the E12.5 embryonic stage. These cell condensations form the anlagen of the future skeleton. At this stage, Runx2 mRNA is expressed exclusively in the developing skeleton and expressed in mesenchymal cells of every anlagen, then Runx2 transcription factor plays central roles in osteoblast differentiation (Ducy *et al.*, 1997). The Runx2 gene has two major transcripts driven by different promoters: Runx2-I, the proximal promoter product, and Runx2-II, the distal promoter product. The translation product of Runx2-I and Runx2-II differ by only 5 and 19 amino acids at their N-terminal regions, respectively. Moreover, Runx2-II has an additional transactivation domain in its N-terminal 19 amino acids. These differences in sequence have been postulated to be functionally important (Thirunavukkarasu *et al.*, 1998). Others have suggested that the two isoforms may not have functional differences. The type II collagen promoter-driven expression of each Runx2 isoform commonly resulted in mineralization of cartilages, suggesting that the two isoforms do not have significant functional differences (Ueta *et al.*, 2001). However, since the expression of two isoforms is regulated by different promoters, their differential expression during development may provide insights into the functional role of the two isoforms. Our previous report demonstrated that Runx2 isoforms are differentially expressed in developing calvaria. Based on their expression patterns, we suggested that Runx2-I plays a role throughout the osteoblast differentiation processfrom early commitment to final differentiation. However, the role of Runx2-II is restricted to later steps in the differentiation process (Park *et al.*, 2001).

Cells expressing Runx2-I have both osteogenic and chondrogenic potential

At the early stages of intramembranous bone formation (E12.5), the primordium of future parietal bone expressed Runx2-I strongly (Figure 2B), which was far from the propagation of the mineralization. At this stage, Col II expression (Figure 2D) was colocalized in sutural mesenchyme in which Runx2-I expression was prominent. These results strongly support the findings of Nah *et al.* (2000), which demonstrated that chick calvarial primordium included a transitional cell population that expressed a subset of chondrogenic markers (Col-II) as well as osteogenic markers (Col-I). They also showed that Col IIA, an alternatively spliced form of Col II, was expressed at this stage. Collectively, these cell populations in the primordium of future parietal bone comprise both osteogenic and chondrogenic potentials. Thus, these Runx2-I and Col-II-positive but Runx2-II-negative cell populations can be depicted as osteo-chondro progenitor cells in the early stages of the skeletogenic process. The expression patterns of both Runx2 isoforms were not very different in other regions of intramembranous bone formation, such as temporal bones, bony maxilla, and the mandible (including the mesenchymal portion of developing tooth buds in the cap stage). These results support our previous notion that Runx2 isoforms are not functionally different in later stages of osteoblast differentiation (Park *et al.*, 2001).

Runx2-I-positive cells support appositional growth of permanent cartilage

Cartilage growth depends on the recruitment of progenitor cells, proliferation, and differentiation of the cells into chondrocytes. Based on the source of the progenitor cells, cartilage growth can be divided into interstitial and appositional growth. In this experiment, the expression pattern of Runx2 isoforms in the

perichondrium was quite different. Runx2-I expression sharply demarcated Col-II positive cartilage, while Runx2-II was not expressed in this region. This pattern of expression was prominent in the nasal septal cartilage. These results suggest that cells expressing Runx2-I in the perichondrium support the appositional growth of the cartilage by providing progenitor cells. Thus, Runx2-I seems to play a pivotal role in early stages of osteogenesis and chondrogenesis. However, since the targeted disruption of Runx2 could not block chondrogenesis (Komori *et al.*, 1997), Runx2 expression in the perichondrium may not be directly related to the commitment of chondrogenesis. In other words, Runx2-I plays a positive role in chondrogenesis but still requires additional factors such as Sox9 (Bi *et al.*, 1999) for the commitment of chondrogenesis. Meanwhile, the Runx2-II expression pattern clearly indicates that it is expressed in cells already committed and undergoing final differentiation step through osteogenesis.

Runx2 is not required for the maintenance of chondrogenic character

As demonstrated in nasal septal cartilage, even though Runx2-I plays a limited role in the commitment of chondrogenesis, Runx2-I expression never overlapped with Col-II expression. In addition, even in the endochondral bone forming process, Runx2-I and Col-II were not colocalized in the proliferating zone in which the cells were believed to maintain the chondrogenic character. These results indicate that the Runx2 isoform is not necessarily required for the cartilaginous character. Furthermore, the elimination of Runx2 activity seemed to be required for the maintenance of chondrogenic character, which is represented by Col-II expression.

The transition of cartilage to bone requires a secondary surge of Runx2-I

During endochondral bone formation, Runx2 expression temporarily disappeared in chondrocytes of the proliferating zone. Its expression appeared again in prehypertrophic or early hypertrophic chondrocytes. Considering that type II collagen promoter-driven Runx2 overexpression strongly enhanced chondrocyte mineralization, and that mineralization is the consequence of chondrocyte hypertrophy (Takeda *et al.*, 2001; Ueta *et al.*, 2001), restoration of Runx2 expression at this stage of chondrocyte differentiation is critical for endochondral bone formation. Furthermore, the ectopic expression of Runx2 in permanent cartilage also resulted in ossification of the tissue (Ueta *et al.*, 2001). These results further support the notion that Runx2 is suppressed to maintain the cartilaginous characteristics of permanent cartilage and epiphyseal growth plate cartilage. Taken together, the spatio-temporal differential expression patterns of Runx2 isoforms in skeletal tissue development could be schematically depicted in Figure 4.

Runx2 isoforms possessing different N-terminal amino acids have been suggested to have different transcriptional activities (Thirunavukkarasu *et al.*, 1998; Harada *et al.*, 1999), though the exact functional differences are unknown. However, a transgenic study indicates that both Runx2 isoforms are not functionally different in terms of chondrocyte maturation to allow for cell hypertrophy and matrix mineralization (Ueta *et al.*, 2001). Despite this solid evidence of the functional redundancy of the Runx2 isoforms, our results indicate that the Runx2-I isoform is responsible for driving the transition of proliferating cartilage to hypertrophic cartilage because only Runx2-I expression was clearly detected in the prehypertrophic zone. Based on mouse genomic sequences, two distinct promoters regulate the transcription of the Runx2 isoforms (Stewart *et al.*, 1997; Harada *et al.*, 1999). Therefore, the differential expression of the two isoforms could be explained as follows; first, having a distinct promoter sequence implies that their transcriptional regulation is regulated by different group of transcription factors. Second, since the first exon of each isoform about 80 kb apart, there is a possibility that their transcription regulatory elements are in different chromatin structures. Thus, the spatio-temporal differential expression pattern of Runx2 isoforms in skeletal tissue development might simply be due to their transcriptional context rather than functional differences. Further studies concerning upstream signals that differentially regulate the expression of two different Runx2 isoforms would be required.

Acknowledgments

This work was supported by Korea Science and Engineering Foundation general research grant (KOSEF 1999-2-209-008-5) and by a grant (CBM-01-A-7) from the Center for Biological Modulators of the 21st Century Frontier R&D Program, the Ministry of Science and Technology, Korea.

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