Continuous expression of *Cbfa1* in nonhypertrophic chondrocytes uncovers its ability to induce hypertrophic chondrocyte differentiation and partially rescues Cbfa1-deficient mice

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Chondrocyte hypertrophy is a mandatory step during endochondral ossification. Cbfa1-deficient mice lack hypertrophic chondrocytes in some skeletal elements, indicating that *Cbfa1* may control hypertrophic chondrocyte differentiation. To address this question we generated transgenic mice expressing *Cbfa1* in nonhypertrophic chondrocytes (*α1(II) Cbfa1*). This continuous expression of *Cbfa1* in nonhypertrophic chondrocytes induced chondrocyte hypertrophy and endochondral ossification in locations where it normally never occurs. To determine if this was caused by transdifferentiation of chondrocytes into osteoblasts or by a specific hypertrophic chondrocyte differentiation ability of Cbfa1, we used the *α1(II) Cbfa1* transgene to restore *Cbfa1* expression in mesenchymal condensations of the Cbfa1-deficient mice. The transgene restored chondrocyte hypertrophy and vascular invasion in the bones of the mutant mice but did not induce osteoblast differentiation. This rescue occurred cell-autonomously, as skeletal elements not expressing the transgene were not affected. Despite the absence of osteoblasts in the rescued animals there were multinucleated, TRAP-positive cells resorbing the hypertrophic cartilage matrix. These results identify Cbfa1 as a hypertrophic chondrocyte differentiation factor and provide a genetic argument for a common regulation of osteoblast and chondrocyte differentiation mediated by Cbfa1.

[*Key Words*: hypertrophic chondrocyte; differentiation; Cbfa1]

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Chondrocytes play critical roles at several stages of endochondral ossification. At the onset of skeletal development, in the areas of the skeleton that will undergo endochondral ossification, undifferentiated mesenchymal cells form condensations that have the shape of the future skeletal elements. Cells within these mesenchymal condensations differentiate into chondrocytes that express specific molecular markers such as *α1(II) collagen*, whereas the remaining undifferentiated mesenchymal cells at the periphery of the condensations form the perichondrium (Horton 1993). Once these cartilaginous models have formed, chondrocytes in their centers further differentiate into hypertrophic chondrocytes. At the time chondrocyte hypertrophy occurs, the perichondrial cells differentiate into osteoblasts to form, around the cartilaginous core, the bone collar (Caplan and Pechak 1987).

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Hypertrophic chondrocytes can be subdivided into two populations: the prehypertrophic chondrocytes that express *α1(II) collagen* predominantly and the hypertrophic chondrocytes proper that express *α1(X) collagen* and become surrounded by a calcified extracellular matrix (Linsenmayer et al. 1991; Poole 1991; Mundlos 1994). Through a vascular endothelium growth factor (VEGF)–dependent pathway the extracellular matrix surrounding hypertrophic chondrocytes favors vascular invasion followed by degradation of the calcified cartilage matrix by chondroclasts (Vu et al. 1998; Gerber et al. 1999). The cartilaginous matrix is then replaced by a bone matrix made mostly of Type I collagen secreted by invading osteoblasts. The ossification process proceeds centripetally, consuming much of the cartilage scaffold. As this cartilaginous front meets the distal ends of a future bone, distal chondrocytes proliferate before they hypertrophy. This zone of proliferating and hypertrophic chondrocytes becomes organized in columns forming the growth plate localized at each end of a bone (Horton 1993). The growth plates will be responsible for linear

skeletal growth. This sequence of events illustrates the pivotal role of chondrocyte hypertrophy as a mandatory step between a cartilaginous scaffold and bona fide bone.

A complex network of regulatory molecules controls the proliferation and/or differentiation of various chondrocyte subpopulations. Early during development the transcription factor Sox9 is required for mesenchymal condensations to form (Bi et al. 1999). Genetic evidence in mouse and human shows that FGF receptors play a critical role in the control of chondrocyte proliferation (Ornitz 2000). Likewise, loss- and gain-of-function experiments have demonstrated that the growth factor parathyroid hormone-related peptide (PTHrP), secreted by cells of the periarticular perichondrium, acts on prehypertrophic chondrocytes to prevent their progression into a hypertrophic phenotype (Karaplis et al. 1994; Lanske et al. 1996; Weir et al. 1996). Indian hedgehog (Ihh), another growth factor secreted by the prehypertrophic chondrocytes, delays chondrocyte hypertrophy through a PTHrP-dependent pathway (Vortkamp et al. 1996; St-Jacques et al. 1999). These studies have established that chondrocyte hypertrophy is under the control of genes expressed in prehypertrophic chondrocytes, but no transcriptional regulator of chondrocyte hypertrophy has been identified.

One transcription factor that may be involved in the control of chondrocyte hypertrophy is Cbfa1. Early during skeletal development and until 12.5 days postcoitum (dpc), *Cbfa1* is expressed at a high level in the cells of the mesenchymal condensations (Ducy et al. 1997). Although *Cbfa1* continues to be expressed at low levels in some chondrocytes beyond 12.5 dpc (Inada et al. 1999; Kim et al. 1999), its expression is largely osteoblast-specific, and it is required for osteoblast differentiation in vivo (Komori et al. 1997; Otto et al. 1997). Analyses of the Cbfa1-deficient mice have indicated that they also have a defective hypertrophic chondrocyte differentiation in some skeletal elements, raising the hypothesis that Cbfa1 may be one regulator of chondrocyte hypertrophy (Inada et al. 1999; Kim et al. 1999). Beyond the control of chondrocyte hypertrophy itself, this is an important question because no transcription factor has been shown to govern the differentiation of both chondrocytes and osteoblasts, two skeletal-specific cell types thought to have a common progenitor (Reddi 1994; Erlebacher et al. 1995).

To assess the role that Cbfa1 may play during chondrocyte hypertrophy, we generated transgenic mice in which *Cbfa1* expression is maintained in nonhypertrophic chondrocytes throughout development. This continuous expression of *Cbfa1* in nonhypertrophic chondrocytes induced ectopic chondrocyte hypertrophy, followed by endochondral ossification. To determine if this was caused by a separate hypertrophic chondrocyte differentiation ability of Cbfa1, we introduced the *α1(II) Cbfa1* transgene in Cbfa1-deficient mice. The transgene induced, in a cell-autonomous manner, chondrocyte hypertrophy and vascular invasion in skeletal elements undergoing endochondral ossification but did not induce osteoblast differentiation. These results identify Cbfa1 as a hypertrophic chondrocyte differentiation factor and provide a genetic argument for a common regulation of osteoblast and chondrocyte differentiation.

Results

Cbfa1 *expression in prehypertrophic and hypertrophic chondrocytes during skeletogenesis*

To define the best experimental approach to study Cbfa1 function during chondrogenesis we analyzed its pattern of expression by in situ hybridization between 14.5 dpc and birth. The identity of the *Cbfa1*-expressing cells was determined by in situ hybridization using as probes *α1(II) collagen*, a marker of proliferating and prehypertrophic chondrocytes; *α1(X) collagen*, a marker of hypertrophic chondrocytes; and *α1(I) collagen*, a marker of osteoblasts (Mundlos 1994). In 14.5-dpc embryos, *Cbfa1* was expressed in prehypertrophic chondrocytes of the scapula at apparently higher levels than in resting and hypertrophic chondrocytes (Fig. 1A). The prehypertrophic chondrocyte identity of these cells was confirmed by the fact that they expressed *α1(II) collagen* but not *α1(I) collagen* (Fig. 1B,C). In long bones of 16.5-dpc embryos the overall level of *Cbfa1* expression in chondrocytes is markedly decreased and restricted to prehypertrophic and hypertrophic chondrocytes in all sections examined (Fig. 1D–G). At birth, *Cbfa1* expression in the growth plate was close to background level (Fig. 1H–K). This analysis demonstrates a progressive disappearance of *Cbfa1* expression in chondrocytes as skeletogenesis proceeds from 14.5 dpc to birth. Moreover, its expression in prehypertrophic chondrocytes is consistent with a role of *Cbfa1* as an inducer of chondrocyte hypertrophy.

Generation of transgenic mice expressing Cbfa1 *in nonhypertrophic chondrocytes throughout development*

In the absence of a transgenic mouse expressing *cre* recombinase only in proliferating and prehypertrophic chondrocytes, we hypothesized that maintaining *Cbfa1* expression in nonhypertrophic chondrocytes beyond 12.5 dpc would allow us to study its function during chondrocyte hypertrophy. We therefore constructed transgenic mice expressing *Cbfa1* under the control of a 3-kb fragment of the mouse *α1(II) collagen* promoter and its chondrocyte-specific enhancer (Zhou et al. 1998) (Fig. 2A). Those mice were termed α1(II) Cbfa1 mice.

Two transgenic mouse lines were obtained. In the first line (line A), male and female transgenic mice had different levels of expression of the transgene (Fig. 2B). In male α1(II) Cbfa1 mice from this line the level of expression of the transgene and of endogenous *Cbfa1* were nearly identical (Fig. 2B). All male α1(II) Cbfa1 mice from line A died at birth. In female α1(II) Cbfa1 mice from line A, the level of expression of the transgene was significantly lower than that of endogenous *Cbfa1* (Fig. 2B). These female mice lived several months and were fertile.

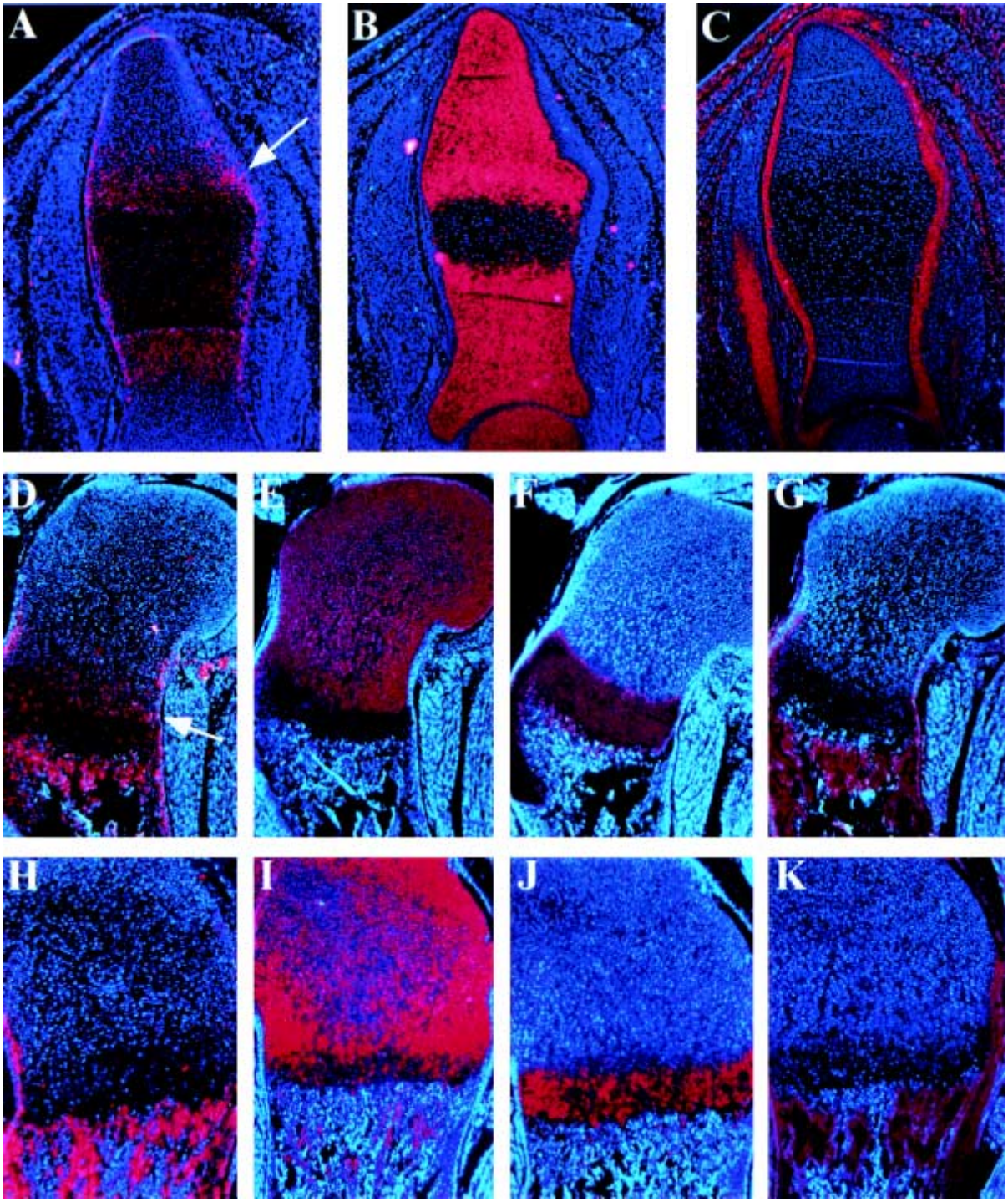

Figure 1. Analysis of *Cbfa1* expression between 14.5 dpc and birth. Adjacent sections of 14.5-dpc (*A–C*), 16.5-dpc (*D–G*), or newborn (*H–K*) wild-type (wt) mouse embryos were hybridized with *Cbfa1* (*A, D, H*), α*1(II) collagen* (*B, E, I*), α*1(X) collagen* (*F, J*), and α*1(I) collagen* (*C, G, K*) probes. Note the decrease in *Cbfa1* expression in chondrocytes as development proceeds (*A, D, H*), and the expression of *Cbfa1* in prehypertrophic chondrocytes (arrows).

Male and female transgenic mice from line A developed the same phenotypic abnormalities that only appeared postnatally in the females. A second transgenic line (line B) was obtained, in which the level of expression of the transgene was similar to the level of expression of endogenous *Cbfa1* in both sexes. All transgenic pups died perinatally with phenotypic abnormalities identical to the one observed in the males of line A. The transgene expression was detected as early as 12.5 dpc and only in cartilage (Fig. 2B,C).

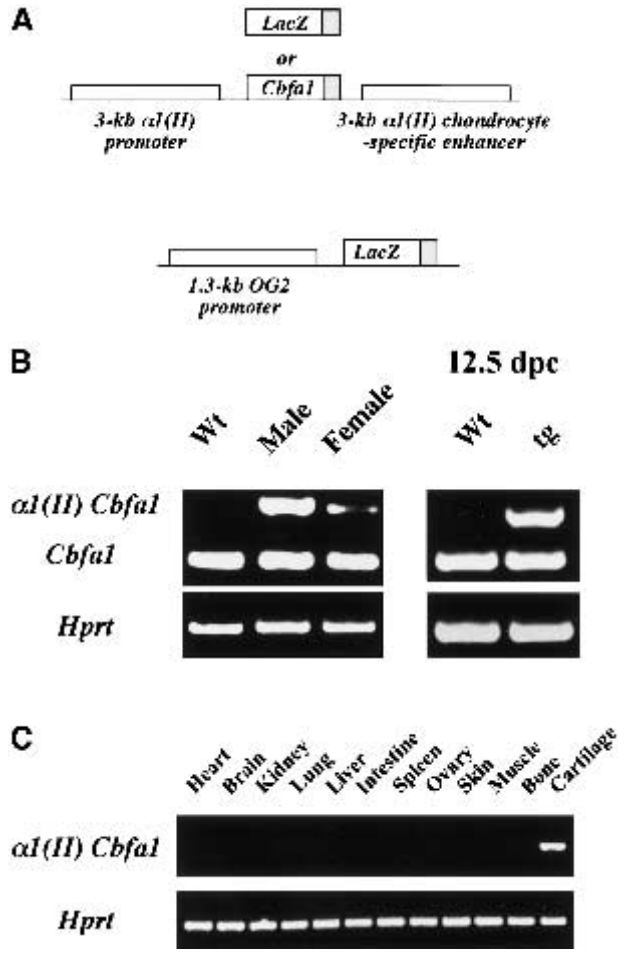

Figure 2. Generation of α1(II) Cbfa1 transgenic mice. (*A*) Schematic representation of the constructs used. Transgenic mice contain either the *Cbfa1* cDNA under the control of a chondrocyte-specific *α1(II) collagen* promoter/enhancer cassette and the *LacZ* gene driven by the *Osteocalcin* promoter, or the chondrocyte-specific cassette driving *LacZ* alone. Gray boxes indicate heterologous polyA regions. (*B*) Comparison of endogenous *Cbfa1* expression and of *α1(II) Cbfa1* transgene expression in wild-type and male and female transgenic mice by RT-PCR. Transgene expression in male transgenic mice is similar to *Cbfa1* expression, whereas in female transgenic mice endogenous *Cbfa1* is expressed at a higher level than the transgene. The transgene is expressed as early as 12.5 dpc. *Hprt* amplification was used as an internal control. (*C*) Cartilage-specific expression of the *α1(II) Cbfa1* transgene. *Hprt* amplification was used as an internal control.

Ectopic hypertrophic chondrocyte differentiation and endochondral ossification in α1(II) Cbfa1 mice

As an initial method to study skeletal cell differentiation we used alcian blue/alizarin red staining of skeletal preparations (McLeod 1980). Alcian blue stains unmineralized cartilaginous matrices, whereas alizarin red stains mineralized cartilaginous and bony matrices. The most striking phenotypic abnormality in the α1(II) Cbfa1 mice was the presence of extensive zones of mineralization in parts of the skeleton where it normally never occurs (Kaufman 1992; black arrowheads in Fig. 3A). These included the chondrocostal cartilage and the trachea, on which we focused our analysis.

In the α1(II) Cbfa1 mice dying at birth (males of line A and all mice of line B), the existence of a mineralized rigid rib cage as well as the shorter circumference of the rib cage prevented lung expansion (Fig. 3A,B). As a result lungs did not contain any air at birth. In contrast, female α1(II) Cbfa1 mice of line A had a normal-appearing rib cage at birth, and their chondrocostal cartilage stained with alcian blue as they should until P7 (data not shown). In 14-d-old transgenic mice, however, small patches of mineralized tissue were visible (Fig. 3C). In 21- and 28-d-old transgenic mice there was a progressive extension of the area staining red (Fig. 3C). To monitor for osteoblast differentiation the *α1(II) Cbfa1* transgene was coinjected with a construct containing a 1.3-kb fragment of the mouse *Osteocalcin gene 2* (*OG2*) promoter, driving the *LacZ* gene (Fig. 2A). This 1.3-kb *OG2* promoter fragment drives the expression of a reporter gene in differentiated osteoblasts in vivo (Frendo et al. 1998). As shown in Figure 3D, the chondrocostal cartilage of 1-mo-old female α1(II) Cbfa1 mice (line A) stained blue following LacZ staining, indicating that there were differentiated osteoblasts in this part of the ribs. This was never observed in wild-type animals (Fig. 3D).

The cellular basis of this phenotype was monitored by histologic analysis of the chondrocostal cartilage. No hypertrophic chondrocyte was observed in wild-type animals at any stage analyzed (Fig. 3I). In contrast, hypertrophic chondrocytes were already present in the chondrocostal cartilage of 16.5-dpc male α1(II) Cbfa1 embryos and at birth in female transgenic mice of line A (Fig. 3E,F). In 14-d-old transgenic mice there was a true growth plate cartilage with resting, proliferating, and hypertrophic chondrocytes (Fig. 3G). In 1-mo-old transgenic mice there were, below the growth plate, bone trabeculae (Fig. 3H) containing alkaline phosphatase-positive osteoblasts and tartrate-resistant acid phosphatase (TRAP)–positive multinucleated osteoclasts (data not shown). Ectopic skeletal mineralization was also observed in the larynx of the α1(II) Cbfa1 mice: The cricoid and thyroid cartilage stained red at birth, and at 2 mo the tracheal rings of transgenic mice contained patches of mineralized areas (Fig. 3J). This was never seen in wt littermates. Histologic examination confirmed the presence of hypertrophic chondrocytes in the thyroid cartilage of newborn α1(II) Cbfa1 mice and in the tracheal rings of 2-mo-old transgenic mice (data not shown). Taken together, these results indicate that continuous expression of *Cbfa1* in nonhypertrophic chondrocytes induces hypertrophic chondrocyte differentiation and endochondral ossification.

Cbfa1 accelerates chondrocyte differentiation in growth plate cartilage

Besides the ectopic endochondral ossification, there was also premature endochondral ossification in other areas of the skeleton such as the chondrocranium (open arrow-

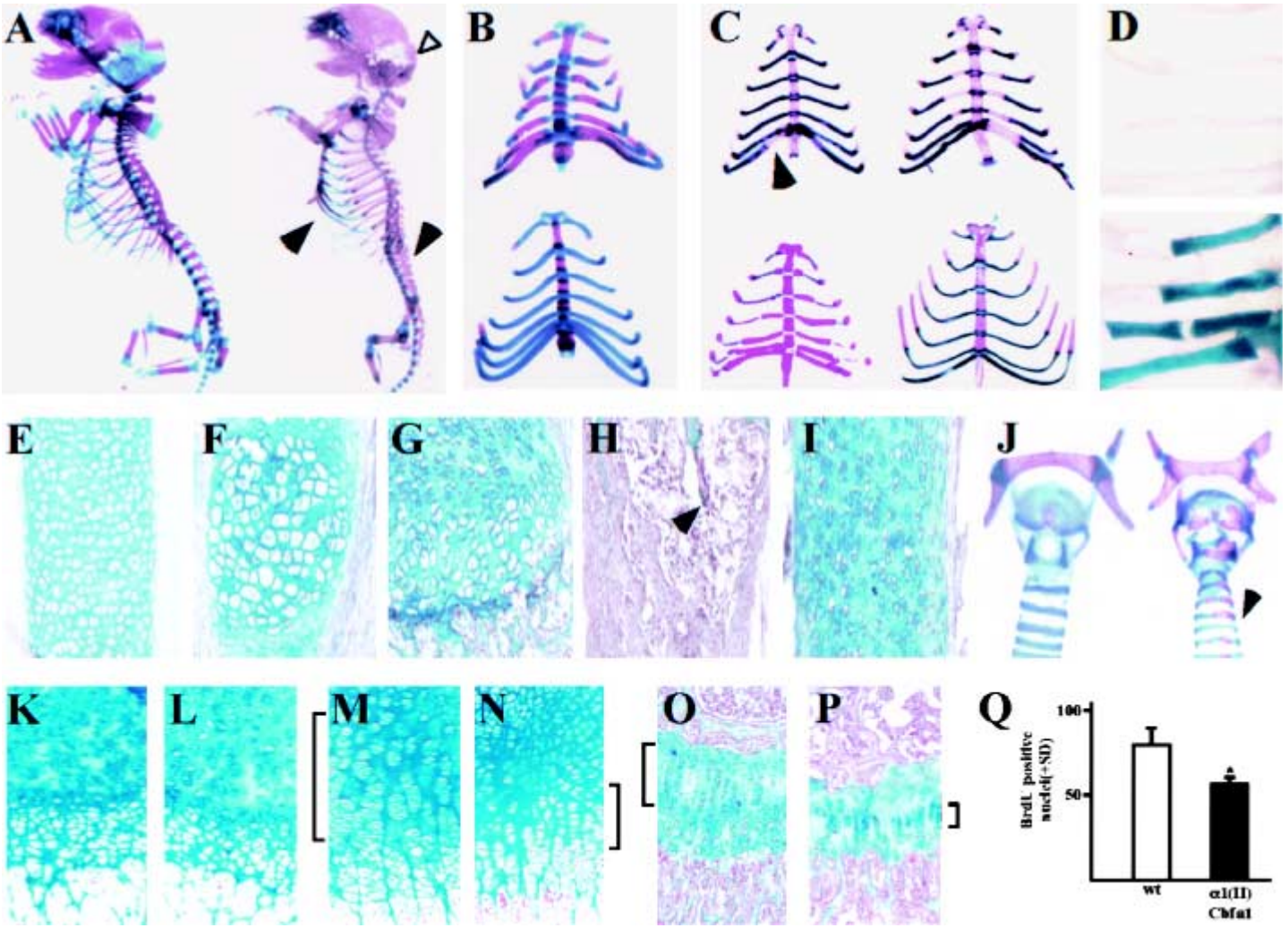

Figure 3. Ectopic and accelerated chondrocyte hypertrophy and endochondral ossification in α1(II) Cbfa1 mice. (*A–C*) Alcian blue/alizarin red staining of skeletal preparations. (*A*) Wild-type (*left*) and α1(II) Cbfa1 male (*right*) mice at birth (P0). Black arrows point at sites of ectopic calcification in transgenic mice; the empty arrow points at the accelerated mineralization of the chondrocranium. (*B*) Rib cages of wild-type (*bottom*) and α1(II) Cbfa1 male mice (*top*) at P0. (*C*) Rib cages of α1(II) Cbfa1 female mice at P14 (*top left*), P21 (*top right*), and P28 (*bottom left*). Note the progressive appearance of mineralized areas (arrowhead) in the transgenic mice, whereas the chondrocostal cartilage of wild-type mice remains unmineralized (*bottom right*, P28). (*D*) LacZ staining showing *Osteocalcin* expression at P28 in the chondrocostal cartilage of the α1(II) Cbfa1 (*bottom*) mice but not of wild-type mice (*top*). (*E–I*) Alcian blue/eosin staining of sections through the chondrocostal cartilage of α1(II) Cbfa1 males 16.5 dpc (*E*), α1(II) Cbfa1 females at P0 (*F*), P14 (*G*), and P28 (*H*), and wild-type mice at P28 (*I*). Hypertrophic chondrocytes (*E–G*), then bone trabeculae (*H*, arrowhead) are present in the transgenic but not in wild-type mice. (*J*) Alcian blue/alizarin red staining of the larynx from wild-type (*left*) and α1(II) Cbfa1 mice (*right*) at 2 mo. Note the mineralization of the tracheal rings in transgenic mice (arrowhead). (*K–P*) Alcian blue/eosin staining of sections through the growth plate of wild-type (*K, M, O*) and α1(II) Cbfa1 (*L, N, P*) mice at P5 (*K, L*), P10 (*M, N*), and P28 (*O, P*). The region of proliferating chondrocyte becomes shorter over time in the α1(II) Cbfa1 mice (brackets). (*Q*) Number of BrdU-positive nuclei per one section of growth plate (asterisk: $P < 0.05$).

head in Fig. 3A) and the long bones. Indeed, α1(II) Cbfa1 mice became growth-retarded over time (data not shown), leading us to study their growth plates. The growth plate cartilage of α1(II) Cbfa1 mice and wild-type littermates were indistinguishable until P5 (Fig. 3K,L). In contrast, at P10 the zone of proliferating chondrocytes was reduced in α1(II) Cbfa1 mice compared to wild-type littermates (Fig. 3M,N). The same abnormality was observed in 28-d-old α1(II) Cbfa1 mice (Fig. 3O,P). To test whether the reduction in size of the areas containing nonhypertrophic chondrocytes was caused by an arrest or by a decrease in chondrocyte proliferation, we performed bromodeoxyuridine (BrdU) labeling. In the α1(II) Cbfa1 mice there were significantly fewer positive cells ($n = 56 \pm 4$) than in wild-type mice ($n = 79 \pm 10$), indicating that chondrocyte proliferation was decreased in α1(II) Cbfa1 mice (Fig. 3Q).

Cbfa1 transactivation function is required to induce chondrocyte hypertrophy

Next we asked whether the ectopic chondrocyte hypertrophy observed in α1(II) Cbfa1 mice required the transactivation function of Cbfa1. We first generated lines of transgenic mice (*α1(II) ΔPST*) expressing a truncated form of Cbfa1 lacking the proline, serine, threonine-rich (PST) domain and one transactivation domain in Cbfa1 (Thirunavukkarasu et al. 1998) (Fig. 4A). We also generated transgenic mice (*α1(II) Cbfa1a*) expressing an alternative-splice form of *Cbfa1* isolated in the initial cloning effort for an osteoblast-specific transcription factor (P. Ducy and G. Karsenty, unpubl.). This Cbfa1 isoform, temporarily called Cbfa1a, was also cloned as a site of proviral insertion (Stewart et al. 1997). The only difference between Cbfa1 and Cbfa1a is that exon 9, encoding most of the PST domain, has been replaced by another exon localized more toward the 3′ end and encoding a different PST-rich domain (Fig. 4B; data not shown). The DNA-binding domains of Cbfa1 and Cbfa1a are identical; therefore, they bind equally well to their recognition sites (data not shown). However, in DNA cotransfection assays Cbfa1a cannot transactivate a vector containing multiple copies of an oligonucleotide containing OSE2, a consensus Cbfa1-binding site, but Cbfa1 can (Fig. 4C). Immunohistochemistry analysis performed in chondrocostal cartilage at P5 showed a similar level of exogenous Cbfa1, ΔPST, and Cbfa1a in the respective transgenic mice, whereas endogenous *Cbfa1* expression could not be detected in wild-type chondrocostal cartilage at that age (Fig. 4D). However, neither α1(II) ΔPST nor α1(II) Cbfa1a mice developed morphological abnormalities of bone and cartilage (Fig. 4D), indicating that the transactivation ability of Cbfa1 is required to induce chondrocyte hypertrophy.

Abnormal expression of molecular markers of chondrocyte hypertrophy in α1(II) Cbfa1 *mice*

To further demonstrate that *Cbfa1* induces chondrocyte hypertrophy, we analyzed the expression of molecular markers of proliferating, prehypertrophic, and hypertrophic chondrocytes in chondrocostal cartilage. In 16.5-dpc wild-type embryos chondrocytes only expressed *α1(II) collagen* (Fig. 5A). In the α1(II) Cbfa1 mice, the level of expression of *α1(II) collagen* was decreased in some ribs (Fig. 5A). *Ihh*, a gene expressed in prehypertrophic chondrocytes, was ectopically expressed, revealing the prehypertrophic chondrocytes identity of some cells in the α1(II) Cbfa1 mice (Fig. 5A). There was also a large area of *α1(X) collagen*–expressing cells, that is, hypertrophic chondrocytes in α1(II) Cbfa1 mice (Fig. 5A). The pattern of expression of these molecular markers was the same in newborn transgenic mice except for a further decrease in *α1(II) collagen* expression and the presence of a small area of *α1(I) collagen*–expressing cells, indicating the beginning of osteogenesis (Fig. 5B).

Phenotypic rescue of the Cbfa1-deficient mice by the α1(II) Cbfa1 *transgene*

To determine whether ectopic endochondral ossification in the α1(II) Cbfa1 mice was caused by transdifferentiation of chondrocytes into osteoblasts or by a specific hypertrophic chondrocyte differentiation ability of Cbfa1, we introduced the *α1(II) Cbfa1* transgene in Cbfa1-deficient mice using female α1(II) Cbfa1 mice of line A. Alcian blue/alizarin red staining of skeletal preparations of Cbfa1-deficient and α1(II) Cbfa1/Cbfa1-deficient newborn mice showed that the transgene rescued the absence of skeletal mineralization, a hallmark of the Cbfa1-deficient mice (Fig. 6A). The shaft of all long bones, ribs, and vertebrae were mineralized (Fig. 6A–C). As was the case with the α1(II) Cbfa1 mice, we observed ectopic mineralization in the chondrocostal cartilage, the base of the skull, and the larynx of these rescued animals (arrowheads in Fig. 6B,C). This rescue was restricted to skeletal areas undergoing endochondral ossification and was never observed in skeletal areas undergoing intramembranous ossification. For instance, the *α1(II) Cbfa1* transgene did not rescue the marked delay in clavicle development or the absence of fontanelle closure observed in Cbfa1 +/– mice (Otto et al. 1997) (Fig. 6E; data not shown). To understand the spatial restriction of this rescue we studied the pattern of expression of the transgene using an *α1(II) promoter/enhancer–LacZ* transgene (Fig. 2A). There was no staining in the calvaria or clavicle that both ossifies through an intramembranous mechanism (Kaufman 1992) and that were not rescued by the *α1(II) Cbfa1* transgene (Fig. 6F; data not shown). The absence of phenotypic rescue of the Cbfa1-deficient mice beyond the zone of expression of the transgene indicates that this rescue occurs through a cell-autonomous mechanism. To compare *Cbfa1* levels of expression between wild-type and α1(II) Cbfa1/Cbfa1-deficient mice, we performed immunohistochemistry in 18.5-dpc embryos digits, a structure that differentiates later than the rest of the skeleton. We observed similar expression of *Cbfa1* in chondrocytes of wild-type and α1(II) Cbfa1/Cbfa1-deficient mice, whereas Cbfa1 was absent in the digits of Cbfa1-deficient embryos (Fig. 6D).

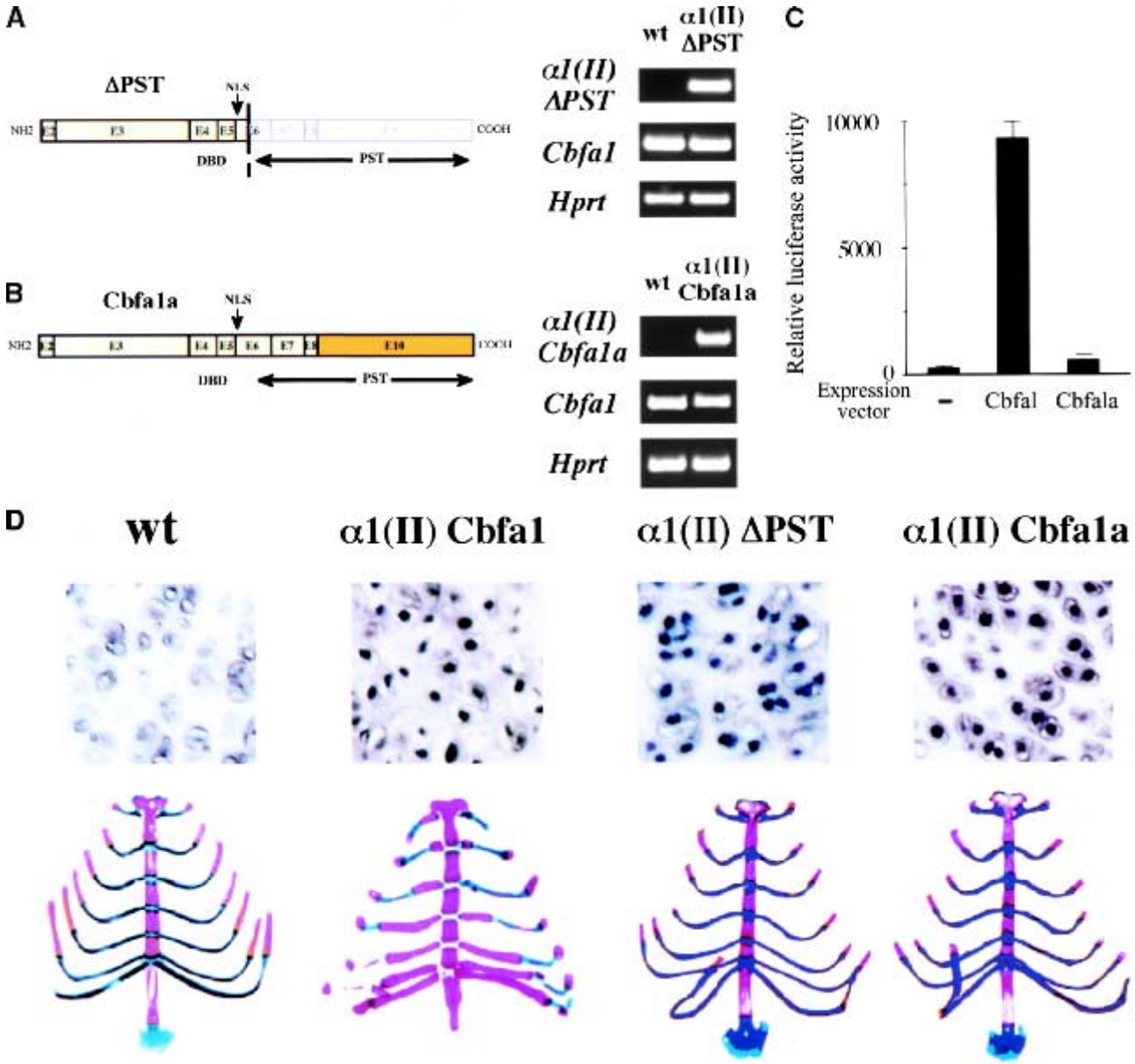

Figure 4. Requirement of Cbfa1 transactivation function to induce chondrocyte hypertrophy. Schematic representation of the (*A*) ΔPST and (*B*) Cbfa1a forms of Cbfa1 used to generate transgenic mice. Yellow boxes represent exons present; gray and gold boxes represent exons missing or replaced, respectively, in the mutated *Cbfa1* cDNAs. The α*1(II) ΔPST* (*A*) and α*1(II) Cbfa1a* (*B*) transgene expressions were confirmed by RT-PCR in respective transgenic mice (*right panels*). (*C*) Absence of transactivation of a reporter construct containing multimerized Cbfa1-binding sites upon cotransfection with a *Cbfa1a* expression vector. (*D*) Immunohistochemistry analysis of chondrocostal cartilage of the α1(II) Cbfa1, α1(II) ΔPST, α1(II) Cbfa1a, and wild-type mice at P5 using an anti-N-terminal Cbfa1 antibody. A similar expression level of each transgenic protein was observed in respective transgenic mice, but there was no expression in wild-type mice. Absence of phenotypic abnormality in transgenic mice expressing either α*1(II) Cbfa1 ΔPST* or α*1(II) Cbfa1a*. Skeletal preparations of rib cages from 1-mo-old animals. NLS, nuclear localization signal.

Hypertrophic chondrocyte differentiation and vascular invasion but no osteoblast differentiation in the α1(II) Cbfa1/Cbfa1-deficient (rescued) mice

To determine if the mineralized skeleton observed in the α1(II) Cbfa1/Cbfa1-deficient mice (hereafter, called rescued mice) was owing to the presence of a calcified cartilaginous matrix only or to the presence of a bony matrix, we performed a histological analysis in 18.5-dpc embryos. As shown in Figure 7, A and B, the rescued mice had hypertrophic chondrocytes in the femur bone, but the Cbfa1-deficient mice (Inada et al. 1999; Kim et al. 1999) had none there, or in any other bones examined (data not shown). The identity of these hypertrophic cells was verified by the study of the expression of mo-

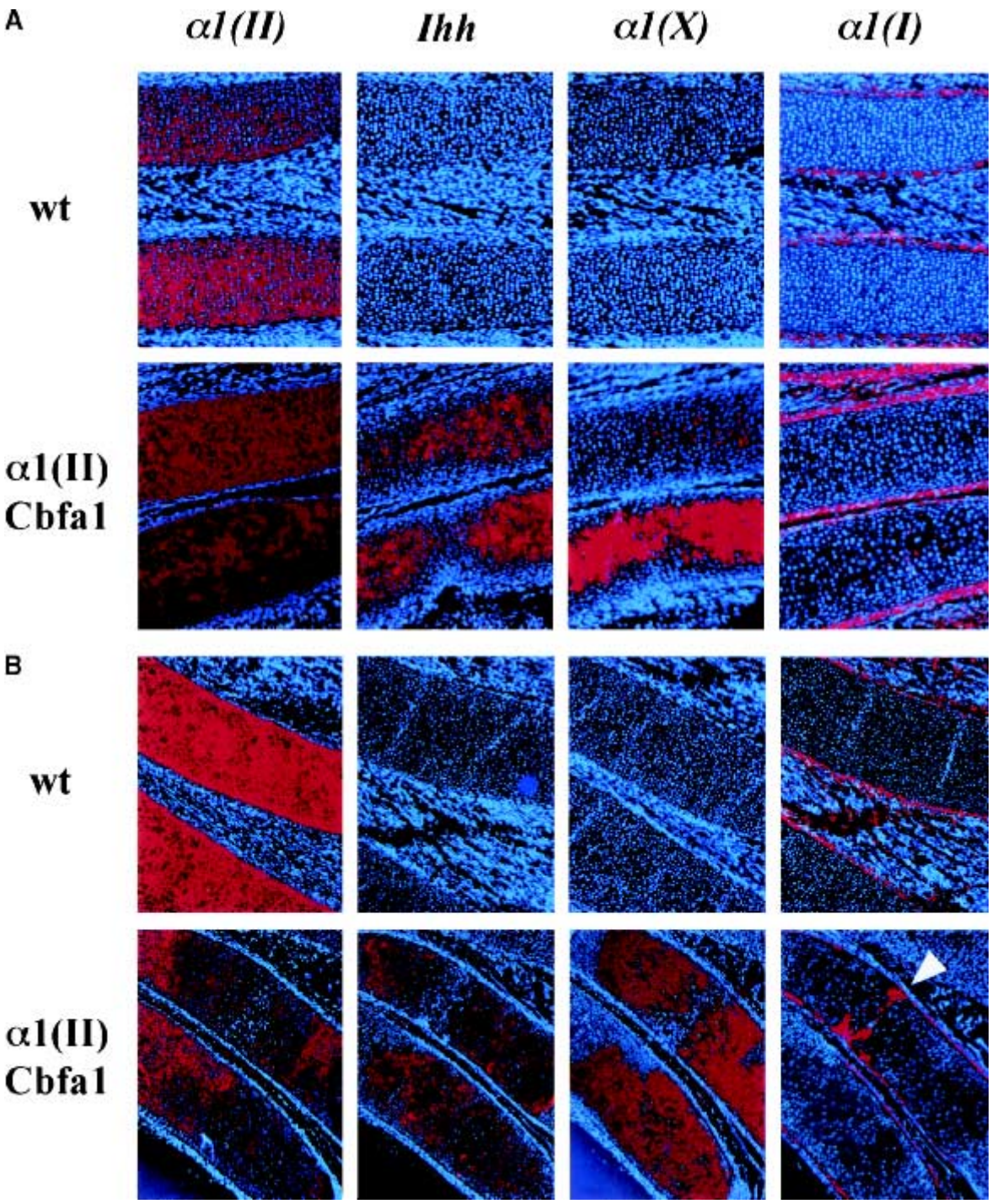

Figure 5. Abnormal expression of molecular markers of chondrocyte hypertrophy in α1(II) Cbfa1 mice. (*A, B*) In situ hybridization analysis of 16.5-dpc embryos (*A*) or newborn mice (*B*). Sections through the chondrocostal cartilage. *α1(II) collagen* (*α1(II)*) expression is decreased in transgenic mice compared to wild-type littermates. *Ihh* and *α1(X) collagen* (*α1(X)*) are only expressed in transgenic mice. Note the *α1(I) collagen* (*α1(I)*) expression in the transgenic cells (white arrowhead in *B*).

lecular markers in 18.5-dpc embryos. *Ihh*, a marker of prehypertrophic chondrocytes that is not expressed in the humeri or femurs of the Cbfa1-deficient mice (Inada et al. 1999; Kim et al. 1999), was expressed in the humeri and femurs of the rescued mice at 18.5 dpc (Fig. 7C; data not shown). The same was true for *α1(X) collagen*, a marker of hypertrophic chondrocytes (Fig. 7C).

Immunohistochemistry analysis showed the presence of VEGF, a protein required for angiogenesis, and a decrease in Chondromodulin-I expression, an endogenous

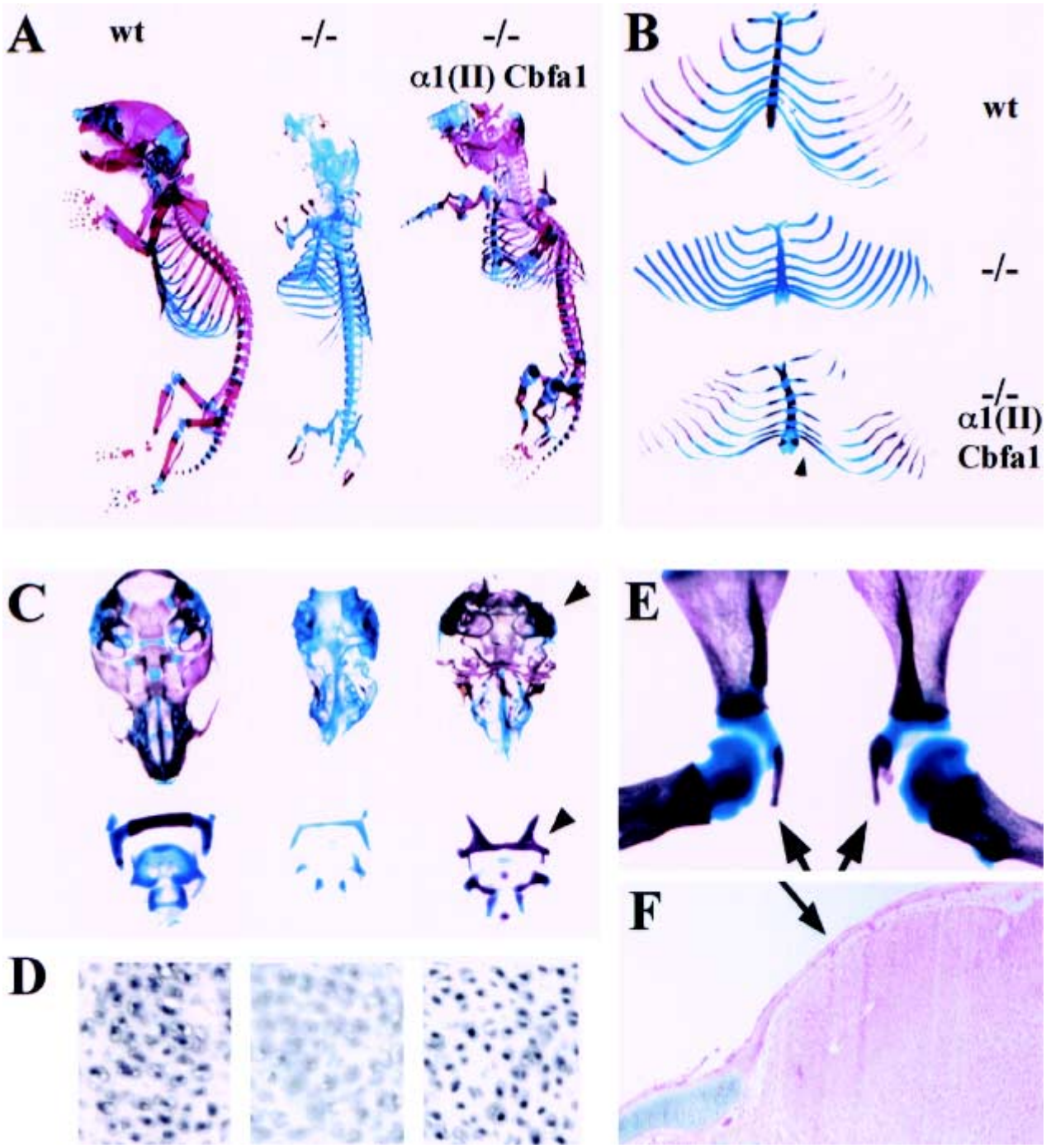

Figure 6. Partial phenotypic rescue of the Cbfa1-deficient mice by the *α1(II) Cbfa1* transgene. Alcian blue/alizarin red staining of skeletal preparations. (*A*) Whole mount preparations of newborn mice. (*B, C*) Magnification of the rib cages (*B*) and the skulls and larynx (*C*). (*D*) Immunohistochemical analysis of digits at 18.5 dpc using an anti-N-terminal Cbfa1 antibody. Cbfa1 protein expression in α1(II) Cbfa1/Cbfa1-deficient mice (*right*) was almost comparable to that of wild-type mice (*left*), but there was no expression in Cbfa1-deficient mice (*middle*). (*E*) Hypoplasia of the clavicle (arrows) in Cbfa1 +/– mice harboring (*right*) or not harboring (*left*) the *α1(II) Cbfa1* transgene. (*F*) Absence of LacZ staining in calvaria (arrow) of an 18.5-dpc α1(II) LacZ transgenic embryo.

inhibitor of angiogenesis (Azizan et al. 2000; Hiraki et al. 1997), in the rescued mice compared to the Cbfa1-deficient mice (Fig. 7D). To further demonstrate that the *α1(II) Cbfa1* transgene expression could induce angiogenesis, we also performed an in vitro angiogenic assay. Chondrocostal cartilage from either wild-type, α1(II) Cbfa1, or α1(II) Cbfa1a transgenic mice was dissected and embedded into collagen gels containing bovine vascular endothelial cells. α1(II) Cbfa1 chondrocostal cartilage, which contains hypertrophic chondrocytes, in-

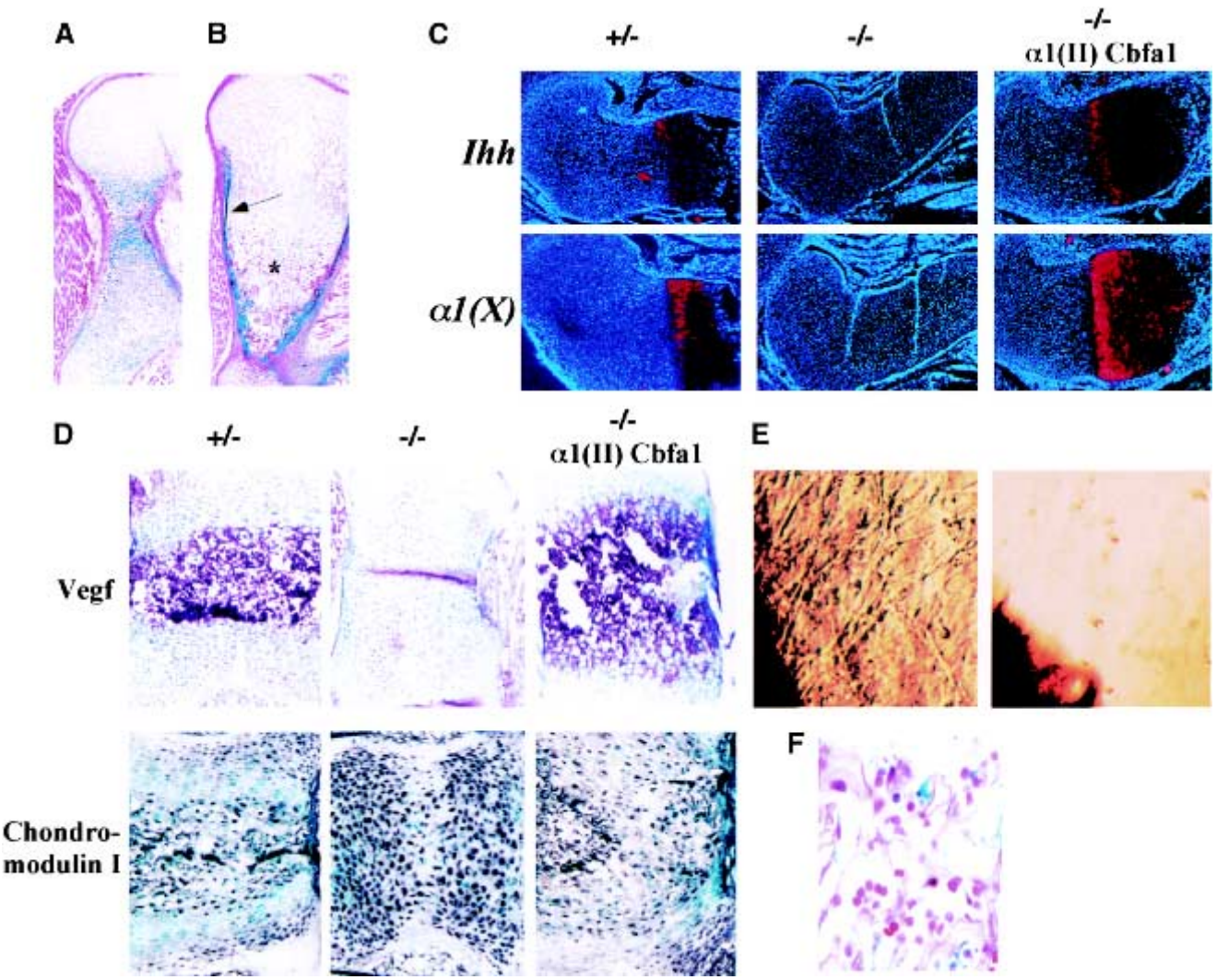

Figure 7. Chondrocyte hypertrophy and vascular invasion in α1(II) Cbfa1/Cbfa1-deficient (rescued) mice. (*A, B*) LacZ/eosin staining of femurs from Cbfa1-deficient mice harboring (*B*) or not harboring (*A*) the *α1(II) Cbfa1* transgene. Hypertrophic chondrocytes (asterisk) are observed only in the presence of the transgene (*B*). (*C*) In situ hybridization analysis of long bones in 18.5-dpc embryos. *Ihh* and *α1(X) collagen* (*α1(X)*)–expressing cells are present in wild-type and rescued mice, indicating their prehypertrophic and hypertrophic chondrocyte nature. (*D*) Immunohistochemistry showing VEGF synthesis and the decreased *Chondromodulin-I* expression in the rescued mice compared to Cbfa1-deficient mice. (*E*) Tube formation assay showing the angiogenic activity of α1(II) Cbfa1 chondrocostal cartilage (*left*), but not of α1(II) Cbfa1a chondrocostal cartilage (*right*). (*F*) Presence of erythrocytes, which is indicative of vascular invasion in femurs of α1(II) Cbfa1/Cbfa1-deficient 19.5-dpc embryos.

duced proliferation and migration of endothelial cells and the formation of tubular structures as previously described (Vu et al. 1998). In contrast, wild-type and α1(II) Cbfa1a chondrocostal cartilage without hypertrophic chondrocytes did not induce migration of endothelial cells or the formation of tubular structures (Fig. 7E; data not shown). The angiogenesis induced by the *α1(II) Cbfa1* transgene led to vascular invasion as illustrated by the presence of erythrocytes in the skeletal elements of the rescued embryos (Fig. 7F).

We made use of the *LacZ* gene inserted in the *Cbfa1* locus of the Cbfa1-deficient mice (Otto et al. 1997) to show by Xgal staining that the *Cbfa1* locus was activated in the bone collar of the rescued mice (Fig. 7B). However, because the *Cbfa1* gene was disrupted by the insertion of *LacZ* in its locus, the Cbfa1 protein was not produced endogenously by the cells of bone collar of either the Cbfa1-deficient mice or the rescued animals (Otto et al. 1997). As a result, there was no bone trabeculae in the rescued mice. The absence of osteoblast in the skeleton of the rescued animals, which was further demonstrated by the absence of *Osteocalcin* expression (Fig. 8A), could only be explained if the transgene was not expressed in the bone collar. To test if this was the case we analyzed the pattern of expression of the *α1(II) collagen–LacZ* transgene. As shown in Figure 8B, there was no LacZ staining in the bone collar of any long bones of 18.5-dpc embryos expressing the *α1(II)–LacZ* transgene. Taking

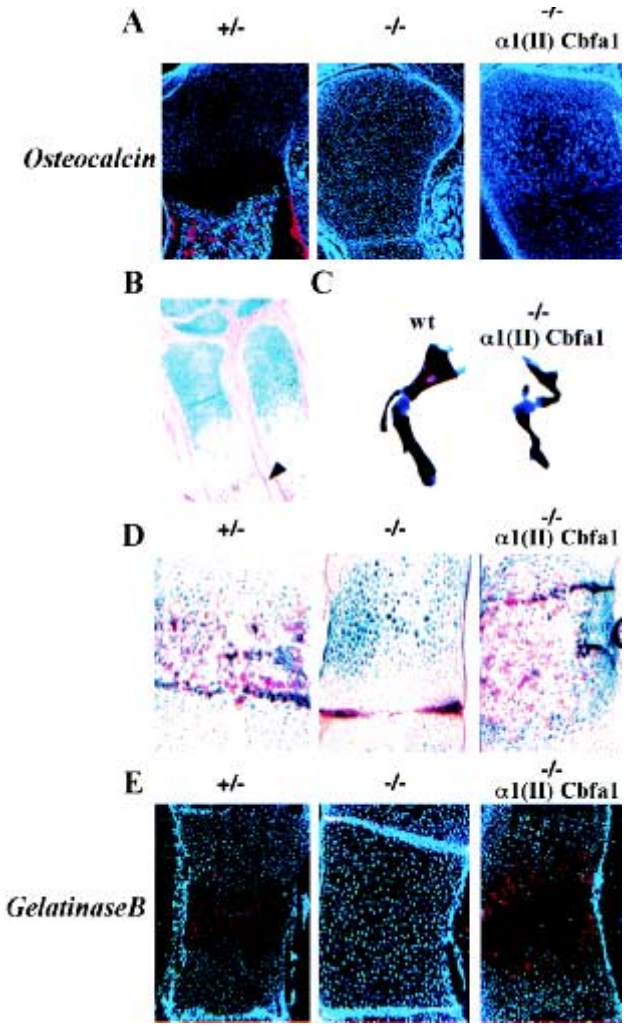

Figure 8. Absence of osteoblasts but existence of cartilage remodeling in the α1(II) Cbfa1/Cbfa1-deficient mice. (*A*) In situ hybridization analysis of long bones of 19.5-dpc embryos. In the Cbfa1-deficient background no *Osteocalcin* expression is detectable whether the transgene is present or not, indicating the absence of osteoblasts in the rescued mice. (*B*) Absence of expression of the *α1(II)–LacZ* transgene in cells of the bone collar of the femur of an 18.5-dpc embryo (arrowhead). (*C*) Alcian blue/alizarin red staining of 19.5-dpc forelimbs. Note the bending of the bone in the rescued mice. (*D*) TRAP-positive (red) multinucleated cells in femurs of α1(II) Cbfa1/Cbfa1-deficient 19.5-dpc embryos. (*E*) In situ hybridization analysis of 18.5-dpc embryo vertebrae showing the expression of *GelatinaseB* in α1(II) Cbfa1/Cbfa1-deficient embryos.

all the data together, our analysis indicates that restoring *Cbfa1* expression in mesenchymal condensations of Cbfa1-deficient mice leads to hypertrophic chondrocyte differentiation, not to transdifferentiation of chondrocytes into osteoblasts.

Cartilage resorption in the rescued mice

The rescued mice had a second phenotypic abnormality: Most of their long bones were bent (Fig. 8C). This was not observed in the Cbfa1-deficient mice and raised the hypothesis that in the rescued animals there was destruction of the cartilaginous matrices. Indeed, skeletal elements of the rescued mice contained TRAP-positive, multinucleated cells (Fig. 8D). These cells also expressed gelatinase B, an enzyme that degrades cartilaginous matrices (Fig. 8E; Vu et al. 1998). The existence of these resorbing cells in a skeleton made only of cartilage suggests that they were similar to the chondroclasts modeling the cartilage in the *GelatinaseB*-deficient mice described by Vu et al. (1998). In the absence of osteogenesis the destruction of cartilage by these cells led to the collapse of the shaft of the future bones and therefore explained their deformations.

Discussion

Our results provide evidence that *Cbfa1* expression in nonhypertrophic chondrocytes is a necessary part of the chondrocyte hypertrophy process during development, at least in some skeletal elements. This is best demonstrated by the fact that skeletogenesis proceeds a step further to reach chondrocyte hypertrophy in Cbfa1-deficient mice. This function is independent of the role of Cbfa1 as an osteoblast differentiation factor and cannot replace it. This demonstrates that Cbfa1 plays multiple roles in skeletogenesis.

Comparison of Cbfa1 *expression in chondrocytes and osteoblasts*

Previous experiments have shown that *Cbfa1* is expressed at a high level until 12.5 dpc of development in cells expressing both *α1(I) collagen*, an osteoblast marker, and *α1(II) collagen*, a chondrocyte marker. On the basis of this coexpression of chondrocyte and osteoblast markers, this cell type was termed osteochondroprogenitor, although evidence of an involvement of Cbfa1 in chondrogenesis was lacking at that time (Ducy et al. 1997). Beyond that stage of development only osteoblast progenitor cells and differentiated osteoblasts maintain such a high level of *Cbfa1* expression. Nevertheless, *Cbfa1* remains expressed at low levels in chondrocytes. A systematic analysis of *Cbfa1* expression in chondrocytes between 14.5 dpc and birth shows that *Cbfa1* is expressed in prehypertrophic chondrocytes and hypertrophic chondrocytes. At birth the level of *Cbfa1* expression in chondrocytes is close to background. Recently Inada et al. (1999) and Kim et al. (1999) also reported *Cbfa1* expression in hypertrophic chondrocytes. Combining our studies demonstrates agreement on one important observation: *Cbfa1* expression precedes chondrocyte hypertrophy, which indicates that it may contribute to hypertrophic chondrocyte differentiation.

Cbfa1 function during chondrogenesis

To study the possible function of Cbfa1 as an inducer of chondrocyte hypertrophy we used the *α1(II) collagen*

promoter/enhancer–-Cbfa1 transgene to prevent the decline of *Cbfa1* expression in nonhypertrophic chondrocytes beyond 12.5 dpc. We also used this transgene to restore *Cbfa1* expression in the skeletal mesenchymal condensations of the Cbfa1-deficient mice.

The analysis of the α1(II) Cbfa1 mice indicates that Cbfa1 does have the ability to induce chondrocyte hypertrophy and endochondral ossification ectopically. *Cbfa1* expression in nonhypertrophic chondrocytes induced the appearance of prehypertrophic chondrocytes expressing *Ihh*. In turn, Ihh expression favors chondrocyte hypertrophy as shown by *α1(X) collagen* expression (St-Jacques et al. 1999), but also induces *Cbfa1* expression in the bone collar (St-Jacques et al. 1999). This, along with the induction of *Vegf* expression in hypertrophic chondrocytes, initiates the cascade of ectopic endochondral ossification observed in these mice. This showed that *Cbfa1* expression is necessary for chondrocytic hypertrophy in some skeletal elements, although there is no evidence at that point that *Cbfa1* expression is sufficient. Our experiments do not exclude the possibility that other factors are required to achieve chondrocyte hypertrophy. Conceivably, Cbfa1 could act in concert with these other factors. Alternatively, they could provide a necessary setting for Cbfa1 permissive action to occur. For instance, because the sensitivity of proliferating chondrocytes to PTHrP prevents their differentiation (Chung et al. 1998), one could hypothesize that *Cbfa1* expression is similarly influenced. We do not know yet whether Cbfa1 induces *Ihh*, *Vegf,* and/or *α1(X) collagen* expression directly or indirectly.

To address whether Cbfa1 causes transdifferentiation of chondrocytes into osteoblasts (Kahn and Simmons 1977; Cancedda et al. 1995; Kim et al. 1999) or, rather, induces chondrocyte hypertrophy, the *α1(II) Cbfa1* transgene was introduced into the Cbfa1-deficient mice (Otto et al. 1997). This transgene induced *Ihh*, *α1(X) collagen*, and *Vegf* expression in skeletal elements where it is not observed in the Cbfa1-deficient mice. As a result, these rescued mice had a fully mineralized skeleton. However, the lack of bone trabeculae and the absence of *Osteocalcin* expression demonstrated that the transgene could not rescue the absence of osteoblasts. This could be expected because on a Cbfa1-deficient background Ihh cannot induce *Cbfa1* expression and osteoblast differentiation in the bone collar, a mandatory step for osteogenesis (St-Jacques et al. 1999). The dissociation between the rescue of the chondrocyte hypertrophy differentiation defect by the *α1(II) Cbfa1* transgene and the absence of osteoblast differentiation demonstrates that Cbfa1 regulates chondrocyte hypertrophy independently of its osteoblast differentiation function.

Three arguments suggest that the control of chondrocyte hypertrophy is a normal function of Cbfa1. First, Cbfa1-deficient mice lack hypertrophic chondrocytes in a few skeletal elements such as humeri and femurs that develop earlier than other elements like the tibia, where chondrocyte hypertrophy does occur normally. Second, this function of Cbfa1 requires the presence of its transactivation domain. Third, the level of expression of the *α1(II) Cbfa1* transgene is not higher than that of endogenous *Cbfa1*, indicating that we are mimicking a true function of Cbfa1 rather than overexpressing it in nonhypertrophic chondrocytes. However, the fact that not all skeletal elements have delayed chondrocyte hypertrophy in the Cbfa1-deficient mice indicates that there are Cbfa1-independent pathways leading to chondrocyte hypertrophy elsewhere in the skeleton. The nature and numbers of these Cbfa1-independent pathways are so far unknown.

Cartilage modeling in absence of bone

The partial rescue of the phenotype of the Cbfa1-deficient mice by the *α1(II) Cbfa1* transgene provides an opportunity to observe the fate of hypertrophic cartilage in an animal unable to produce bone. We observed multinucleated TRAP-positive cells, either osteoclasts or chondroclasts (Vu et al. 1998), in the rescued animals but not in the Cbfa1-deficient mice. The expression of *Vegf* and the presence of erythrocytes in the skeletal elements in the rescued mice indicate that angiogenesis did occur. The abnormal shape of the bones in rescued mice reveals that the multinucleated cells remove the matrix produced by hypertrophic chondrocytes at the time of vascular invasion. The existence of these cells in the absence of any detectable osteoblasts indicates that the differentiation of multinucleated cells able to resorb cartilaginous matrices is independent of osteoblast differentiation (Vu et al. 1998).

Toward a genetic understanding of chondrocyte hypertrophy

Our data together with those of others (Vortkamp et al. 1996; Chung et al. 1998; St-Jacques et al. 1999) begin to unravel a complex genetic network controlling chondrocyte hypertrophy. Our study indicates that *Cbfa1* is located upstream of *Ihh* during chondrogenesis, as shown by the induction of *Ihh* expression ectopically in the *α1(II) Cbfa1* transgenic and in the rescued mice. The absence of *Ihh* expression in some skeletal elements of the Cbfa1-deficient mice (Inada et al. 1999; Kim et al. 1999) also indicates that Cbfa1 is only one of several upstream regulators of *Ihh* expression. Once *Ihh*-expressing prehypertrophic chondrocytes have differentiated, hypertrophy occurs, an event that requires Cbfa1 transactivity function in some skeletal elements. However, *Cbfa1* may also be located downstream of *Ihh*. *Ihh* is likely to control, directly or indirectly, *Cbfa1* expression in the cells of the bone collar and thereby osteoblast differentiation. Moreover, as *Ihh* induces *PTHrP* expression (Lanske et al. 1996; Vortkamp et al. 1996), it is conceivable that *PTHrP* in turn affects *Cbfa1* expression during chondrogenesis when its expression decreases.

In summary, this study provides evidence for a more complex role for Cbfa1 during skeletogenesis than originally anticipated. The ability of Cbfa1 to induce chondrocyte hypertrophy in wild-type and in Cbfa1-deficient

mice indicates that Cbfa1 is one of the transcription factors required for this function. This, along with its well-established role in osteoblast differentiation (Komori et al. 1997; Otto et al. 1997), identifies Cbfa1 as a transcription factor regulating both chondrocyte and osteoblast differentiation, two cell types long thought to share a common progenitor (Reddi 1994; Erlebacher et al. 1995).

Materials and methods

Generation of transgenic mice

α*1(II) Cbfa1*, α*1(II) Cbfa1a*, and α*1(II)* Δ*PST* transgenes were generated by subcloning full-length *Cbfa1* or full-length *Cbfa1a* cDNAs, or PST deletion mutant of *Cbfa1* cDNA (Thirunavukkarasu et al. 1998), respectively, between a 3-kb fragment of the α*1(II)* promoter and its 3-kb chondrocyte-specific enhancer region (Zhou et al. 1998). The *1.3kbOG2–LacZ* construct was generated by subcloning a 1.3-kb fragment of the mouse *OG2* promoter (Ducy and Karsenty 1995) into pLacF (Mercer et al. 1991). Transgenic founders were generated by pronuclear injection according to standard techniques (Bonnerot and Nicolas 1993). The α*1(II) Cbfa1*, α*1(II) Cbfa1a*, and α*I(II)* Δ*PST* transgenes were, respectively, coinjected with the *1.3kbOG2–LacZ* construct. Genotypes were determined by PCR using as primers: 5′-GGCAGCACGCTATTAAATCCAA-3′ and 5′-GGTTT CAGGGGGAGGTGTGGGAGG-3′ for the α1(II) Cbfa1 mice; 5′-CTGGACATCATAGCAAAGGCCC-3′ and 5′-GGTTTCA GGGGGAGGTGTGGGAGG-3′ for the αI(II) Cbfa1a mice; and 5′-CGGAGCGGACGAGGCAAGAGTTTC-3′ and 5′-GGTTT CAGGGG GAGGTGTGGGAGG-3′ for the αI(II) ΔPST mice. Sex was determined by PCR using the *Sry*-specific primers 5′-CATGACCACCACCACCACCAA-3′ and 5′-TCATGAGACT-GCCAAC CACAG-3′ (Jeske et al. 1996).

RT-PCR analysis

Total RNA was prepared from 12.5-dpc embryos or newborn mice (n = 3 for each genotype). RNA extraction, cDNA synthesis, and PCR amplification were performed using standard protocols (Ausubel et al. 1995). Exon 2 amplification of the HPRT gene was used as an internal control of the quantity and quality of the cDNAs. The following sets of primers were used: for α*I(II)* transgenes, 5′-CCAGGCAGTTCCCAAGCATT-3′ and 5′-AGAGCTATGACGTCGCATGCACAC-3′; for endogenous *Cbfa1*, 5′-GGCAGCACGCTATTAAATCCAAA-3′ and 5′-TG ACTGC CCCCACCCTCTTAG-3′; and for *Hprt*, 5′-GTTGA GAGATCATCTCCACC-3′ and 5′-AGCGATGATGAACCAG GTTA-3′.

Skeletal preparation

Mice were dissected, fixed in 100% ethanol overnight, then stained in alcian blue dye followed by alizarin red solution according to standard protocols (McLeod 1980). At least 4 mice of each genotype were analyzed for each stage.

Histology and in situ hybridization

Tissues were fixed in 4% paraformaldehyde/PBS overnight at 4°C and decalcified in 25% EDTA at 37°C for 3 d when older than newborn. Specimens were embedded in paraffin and sectioned at 6 μm. For histological analysis sections were stained with alcian blue (1% alcian blue 8GX, 3% acetic acid) and counterstained with eosin. TRAP staining was done with pararosaniline (Sigma) following established conditions (Bronckers et al. 1996). In situ hybridization was performed using ^{35}S-labeled riboprobes. The *Cbfa1*, α*I(II) collagen*, and *Osteocalcin* probes have been previously described (Ducy et al. 1997). The *Ihh* probe is a 540-bp fragment of the *Ihh* 3′ untranslated region. The α*I(X) collagen* probe was obtained from B.R. Olsen (Harvard Medical School). The *GelatinaseB* probe was obtained from Z. Werb (University of California, San Francisco). Hybridizations were performed overnight at 55°C, and washes were performed at 63°C. Autoradiography and Hoechst 33528 staining were performed as described (Sundin et al. 1990). Three mice of each genotype were analyzed for each stage.

LacZ staining, immunohistochemistry, and BrdU labeling

Skinned and eviscerated animals were fixed in 1% paraformaldehyde, 0.2% glutalaldehyde in phosphate buffer at pH 7.3 and stained overnight with X-Gal (5-bromo-4-chloro-3-indoyl β-D-galactosidase). Specimens were embedded in paraffin, sectioned at 6 μm, and counterstained with eosin. Immunohistochemistry was performed according to standard protocol (Ausubel et al. 1995). The anti-N-terminal-Cbfa1 antibody was obtained from A.G. Geiser (E. Lilly). The anti-VEGF antibody was purchased from Santa Cruz. The anti-Chondromodulin-I antibody was obtained from P.J. Neame (University of South Florida, Tampa). Three mice of each genotype were analyzed. For BrdU labeling, mice were injected intraperitoneally with 10^{-4} mM BrdU/g body weight 1 h before sacrifice. Specimen were embedded in paraffin as above. BrdU was detected using a Zymed kit following the manufacturer's protocol (Zymed). BrdU-positive cells present in the growth plate of at least five different sections were counted for both wild-type and αI(II) Cbfa1 mice. Statistical differences between groups were assessed by Student's *t*-test.

Endothelial tube formation assay

The endothelial tube formation assay was performed as previously described (Vu et al. 1998).

DNA transfection assays

F9 cells were transfected with 5 μg of empty, *Cbfa1*, or *Cbfa1a* expression vector (Ducy et al. 1997); 5 μg of p6OSE2-luc reporter vector (Ducy and Karsenty 1995); and 2 μg of pSVβgal plasmid. Transfections, luciferase assays, and β-galactosidase assays were performed as described (Ducy and Karsenty 1995). Data represents ratios of luciferase/β-galactosidase activities, and values are means of six independent transfection experiments.

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