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Mechanisms of Development 106 (2001) 97–106

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Tissue specific regulation of VEGF expression during bone development requires Cbfa1/Runx2

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Received 4 December 2000; received in revised form 9 May 2001; accepted 9 May 2001

Abstract

Vascular endothelial growth factor (VEGF) is a critical regulator of angiogenesis during development, but little is known about the factors that control its expression. We provide the first example of tissue specific loss of VEGF expression as a result of targeting a single gene, Cbfa1/Runx2. During endochondral bone formation, invasion of blood vessels into cartilage is associated with upregulation of VEGF in hypertrophic chondrocytes and increased expression of VEGF receptors in the perichondrium. This upregulation is lacking in Cbfa1 deficient mice, and cartilage angiogenesis does not occur. Finally, over-expression of Cbfa1 in fibroblasts induces an increase in their VEGF mRNA level and protein production by stimulating VEGF transcription. The results demonstrate that Cbfa1 is a necessary component of a tissue specific genetic program that regulates VEGF during endochondral bone formation.

Keywords: Hypertrophy; Chondrocytes; Vascular endothelial growth factor; Angiogenesis; Transcription; Bone development; Endochondral ossification; Vascular endothelial growth factor receptors; Transcription factors; Hypoxia; Transfection; Promoter; Real-time polymerase chain reaction; In situ hybridization

1. Introduction

Angiogenesis is an essential component of normal embryonic development, requiring complex interactions between endothelial cells and cells in the surrounding tissue. Signaling by vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) play key roles in these interactions (Ferrara, 1999; Neufeld et al., 1999). The expression of VEGFR-1 and VEGFR-2 is predominantly restricted to endothelial cells in blood vessels (Carmeliet and Collen, 1999; Eriksson and Alitalo, 1999), whereas VEGFR-3 is found on lymphatic endothelium (Kaipainen et al., 1995). VEGF binds to these receptors and stimulates endothelial cell proliferation, migration and survival. Targeted inactivation of VEGF and its receptors in mice causes abnormalities that reflect their importance in development. The loss of a single VEGF-A allele is sufficient to cause embryonic lethality between E11 and E12 (Carmeliet et al., 1996; Ferrara et al., 1996) as a result of severe cardiac and vascular anomalies, underdevelopment of the forebrain region and defects in the vascularity of other tissues and organs, including the placenta and the nervous system. VEGFR-2 null mice die between E8.5 and E9.5, and have defects in the differentiation of hematopoietic and endothelial cells (Shalaby et al., 1997, 1995); in VEGFR-1 null embryos, endothelial cells are formed, but normal blood vessels do not develop (Fong et al., 1995).

The critical involvement of VEGF in developmental processes suggests a need for tight regulation of its expression. Relatively little is known about the control of VEGF expression, but oxygen tension and a number of cytokines, hormones, and growth factors, such as epidermal growth factor (EGF), transforming growth factor-β (TGF-β), interleukin (IL)-1, IL-6, insulin-like growth factor (IGF)-1 and insulin, are clearly involved in its induction (Cohen et al., 1996; Goad et al., 1996; Goldman et al., 1993; Jingushi et al., 1995; Li et al., 1995; Shinar et al., 1993; Shweiki et al., 1992; Zelzer et al., 1998). The transcription factor, hypoxia inducible factor-1 (HIF-1), represents a key regulator since it has

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0925-4773/01/$ - see front matter
PII: S0925-4773(01)00428-2

been shown to induce the transcription of VEGF in response to several of the above stimuli, including hypoxia, insulin, and IGF-1 (Semenza and Wang, 1992; Zelzer et al., 1998). Surprisingly, however, HIF-1α null embryos were found to express increased VEGF mRNA levels when compared with wild-type embryos. Furthermore, the vascular defects in HIF-1α null embryos did not result from VEGF deficiency, but from mesenchymal cell death (Kotch et al., 1999). These observations suggest the existence of other molecular pathways that regulate VEGF during embryonic development.

In the developing and growing skeleton, VEGF has been shown to play an important role in regulating angiogenesis. For example, in 24-day-old mice, injections of a soluble chimeric VEGF receptor protein, Flt-(1-3)-IgG, inhibited blood vessel invasion into the hypertrophic zone of long bone growth plates and resulted in impaired trabecular bone formation and an expansion of the hypertrophic zone (Gerber et al., 1999). Moreover, there was a concomitant dramatic decrease in the expression levels of the receptors for VEGF. Conditional deletion in the mouse of a single VEGF-A allele in cells expressing collagen II (the predominant collagen synthesized by chondrocytes) resulted in lethality around E10.5 in the majority of embryos because of defects in multiple essential organs. However, a small percentage of these mice survived until E17.5, at which time, impaired vascularization of developing bones could be observed. This provides further support for a connection between bone development and VEGF expression (Haigh et al., 2000).

Skeletal development in vertebrates, with the exception of bones in the cranial vault, jaws, and part of the clavicle, occurs primarily by the process of endochondral bone formation (Olsen et al., 2000). During endochondral bone formation, condensation of mesenchymal cells occurs at the sites of future bones. Subsequent chondrocytic differentiation is followed by growth as the chondrocytes proliferate and produce extracellular matrix. As development proceeds, chondrocytes in the centers of the cartilage templates (anlagen) cease to proliferate and further differentiate to hypertrophy. Hypertrophy is characterized by an increase in cellular volume with the expression of specific matrix components, including collagen type X, a commonly used marker for this state (Iyama et al., 1991; Ninomiya et al., 1986). The maturation of chondrocytes to hypertrophy is followed by rapid invasion of blood vessels, osteoclasts and other mesenchymal cells from the perichondrium into the cartilage which is progressively eroded and replaced by bone marrow and trabecular bone (the primary ossification center).

In this paper, we present evidence for the existence of a genetic program that regulates VEGF gene expression in ossification centers of developing endochondral bones, with the transcription factor Cbfa1/Runx2 as a major component. Cbfa1, a member of the runt-domain transcription factor family, has an essential role in osteoblast differentiation (Karsenty, 2000). Cbfa1 $-/-$ mice have no bones, but an almost perfectly patterned skeleton composed entirely of cartilage (Komori et al., 1997; Otto et al., 1997). In addition, chondrocyte differentiation to hypertrophy is impaired; occurring (after a delay) only in the distal long bones of the appendicular skeleton (tibia–fibula, radius–ulna) and in other small isolated foci. Moreover, interestingly, no blood vessel invasion is apparent in these Cbfa1 null mice, even in the areas where hypertrophic cartilage does develop (Inada et al., 1999; Kim et al., 1999).

The lack of any blood vessel invasion into hypertrophic cartilage in Cbfa1 deficient mice suggests a link between Cbfa1 and VEGF expression. By comparing VEGF gene expression in wild-type and Cbfa1 deficient mice, we find that the upregulation of VEGF that is observed in the hypertrophic zones of growth plates in wild-type mice is absent in the hypertrophic cartilage of Cbfa1 deficient mice. Furthermore, while upregulation of VEGF in the hypertrophic cartilage of wild-type mice is associated with the expression of the VEGF receptors, VEGFR-1 and VEGFR-2, in perichondrial/periosteal vascular endothelial cells, such expression is not observed in the perichondrium of Cbfa1 deficient mice. Finally, we show that over-expression of Cbfa1 in cultured cells increases their VEGF mRNA and protein production by increasing VEGF transcription.

2. Results

2.1. Zones of hypertrophic chondrocytes in Cbfa1 deficient mice are not vascularized

As previously shown, chondrocytes in the diaphyses of the distal long limb bones of Cbfa1 null mice undergo maturation to hypertrophy by E17.5, based on histological appearance and the presence of markers such as collagen X (Inada et al., 1999; Kim et al., 1999). Collagen X expression at E17.5 in the tibia and fibula is observed over a broad domain with decreased expression in the most mature hypertrophic chondrocytes at the center of the hypertrophic zone (Fig. 1I(C,c)). This stage of development in E17.5 Cbfa1 $-/-$ mice resembles stage E15 of wild-type mice (Fig. 1I(A,a)). In order to document blood vessel invasion into the cartilage precisely, we stained for PECAM (CD31), a specific endothelial cell marker. At E17.5 in wild-type mice, abundant CD31 staining can be seen throughout the diaphysis and in the capillary network adjacent to the growth plate (Fig. 1II(A)), while in Cbfa1 deficient animals, there is clearly no vessel invasion into cartilage, although there is abundant vascularization of the perichondrium and the surrounding tissue (Fig. 1II(B,C)). The finding of apparently normal vascularization of the perichondrium in Cbfa1 deficient mice is compatible with the notion that the failure of blood vessels to invade the hypertrophic zone may be caused by the lack of a local angiogenic signal.

2.2. VEGF is not upregulated in tibias of Cbfa1 deficient mice

VEGF expression has been shown to be upregulated in

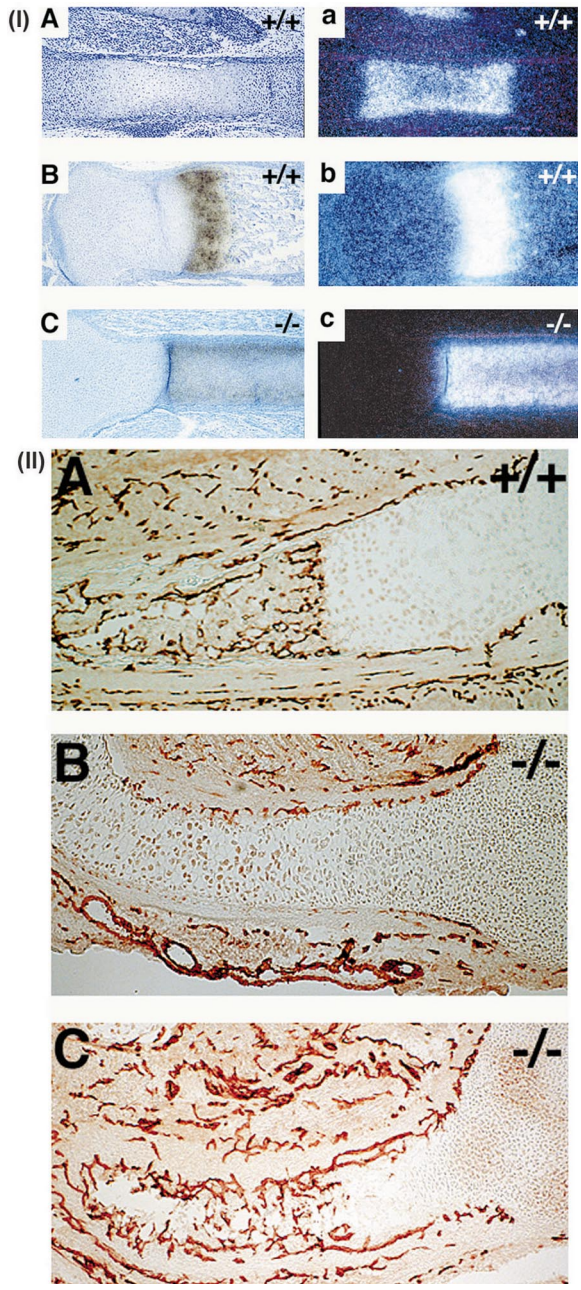

Fig. 1. (I) Expression of collagen X in tibias of wild-type and Cbfa1−/− mice. Sections were hybridized with a ^{33}P-labeled collagen X probe and counterstained with toluidine blue. Panels labeled with capital letters, bright field; panels labeled with small letters, dark field. (A,a) In the tibia of the wild-type mouse at E15, collagen X is expressed in most of the diaphysis. (B,b) Later at E17.5, in the wild-type mouse, expression is localized to the hypertrophic zones of the growth plate. (C,c) In Cbfa1 null mice at E17.5, collagen X expression is seen in most of the diaphysis, an appearance quite similar to wild-type mice at E15 in (A) and (a). (II) CD31 immunostaining of wild-type and Cbfa1−/− tibias at E17.5. (A) In wild-type tibias, there are vessels throughout the periosteum, the surrounding tissue and in the marrow cavity. The cartilage is free of blood vessels. (B) In Cbfa1 deficient mice, the appearance of vessels in the perichondrium and the surrounding tissue is normal, but the entire cartilaginous shaft is devoid of vessels. (C) Tangential section through the perichondrium around the diaphysis of Cbfa1−/− mice showing an extensive network of blood vessels.

hypertrophic chondrocytes of the growth plate, and inhibition of VEGF function impairs cartilage vascularization (Carlevaro et al., 2000; Gerber et al., 1999; Haigh et al., 2000). We therefore studied VEGF expression in the tibia of wild-type and Cbfa1 deficient mice. In wild-type mice at E15, when blood vessel invasion begins, VEGF is co-localized with collagen X in the hypertrophic zone in a broad domain that includes most of the diaphysis (Figs. 1I(A,a) and 2(A,a)). Later at E17.5, when blood vessels have already penetrated the cartilage and marrow is established, VEGF expression is maintained in the hypertrophic zones of the growth plates at both ends of the growing bone (Fig. 2(B,b)). At E17.5, we also observed upregulated VEGF expression in the hypertrophic zones of vertebrae, metatarsals, and ribs (Fig. 2(C,c,D,d,E,e)).

In contrast to the upregulation of VEGF gene expression observed in the wild-type mice, we failed to observe VEGF upregulation in tibias of Cbfa1 deficient mice at E17.5 (Fig. 3(B,b)). The lack of VEGF upregulation in hypertrophic

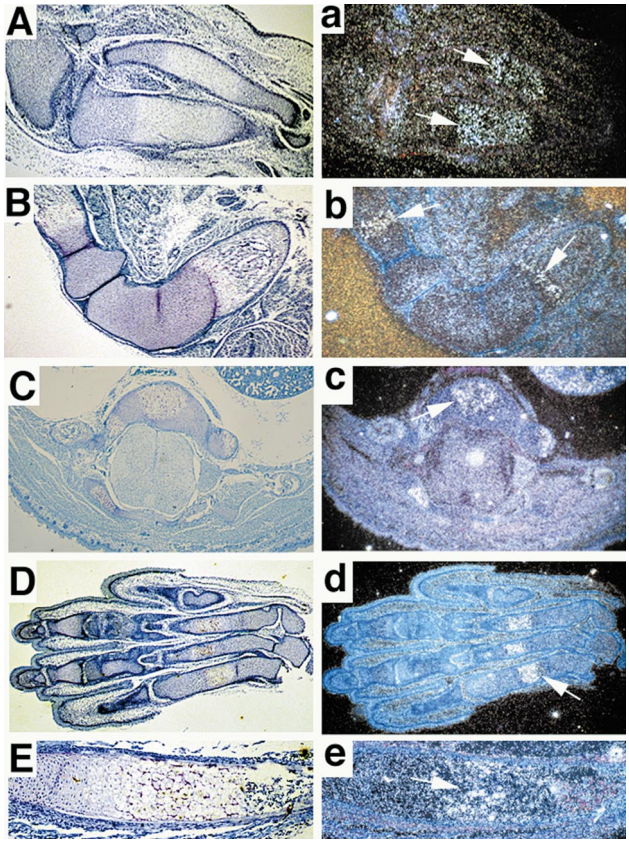

Fig. 2. VEGF expression in wild-type bones. Sections were hybridized with a ^{33}P-labeled VEGF probe and counterstained with toluidine blue. Panels labeled with capital letters, bright field; panels labeled with small letters, dark field. (A,a) E15 tibia and fibula showing signal in a broad zone of hypertrophic chondrocytes (arrows). (B,b) At E17.5, the signal is confined to the hypertrophic zone of the growth plate. The distal femur is in the center and the proximal tibia is in the upper left of the illustration. VEGF expression at E17.5 in hypertrophic zones in vertebral (C,c), metatarsals (D,d) and rib (E,e).

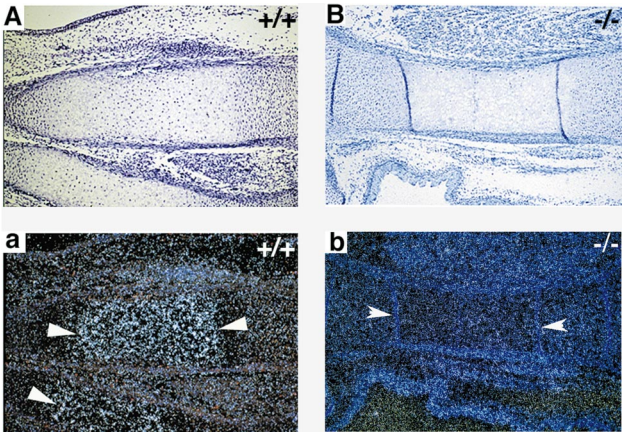

Fig. 3. Lack of VEGF expression in hypertrophic chondrocytes of Cbfa1−/− mice. Sections were hybridized with a ^{33}P-labelled VEGF probe and counterstained with toluidine blue. Panels labeled with capital letters, bright field; panels labeled with small letters, dark field. E15 wild-type tibia is compared with Cbfa1 null tibia at E17.5 because of the striking similarity (see text). (A,a) VEGF expression in the tibia and fibula of wild-type mice at E15 (10× magnification). There are sharply defined expression domains (arrowheads) corresponding to the hypertrophic zones. (B,b) VEGF expression in the tibia of a Cbfa1 null mouse at E17.5 (10× magnification). In the broad zone of hypertrophic chondrocytes found in tibias of Cbfa1−/− mice at this stage, only background labeling is present. The arrowheads in the dark field view bracket the hypertrophic zone.

chondrocytes of Cbfa1 deficient mice suggests that Cbfa1 is necessary for VEGF expression in hypertrophic cartilage.

2.3. Expression of VEGF receptors in Cbfa1 deficient mice

The exposure of endothelial cells to VEGF induces upregulation of both VEGF receptors VEGFR-1 and VEGFR-2 on their surface (Barleon et al., 1997; Shen et al., 1998). Therefore, we examined the expression of VEGFR-1 and VEGFR-2 during blood vessel invasion into developing cartilage (Fig. 4I,II)). In wild-type mice at E15, blood vessels could be seen penetrating into the middle of the hypertrophic zone. At this stage, on the internal side of the overlying perichondrium/periosteum we observed upregulation of both VEGFR-1 and VEGFR-2 gene expression (Fig. 4I(A,a),II(A,a)). This upregulation was most dramatic at the interface between the perichondrial cells and the terminal hypertrophic chondrocytes. Later at E17.5, when blood vessels had fully penetrated the cartilage, the expression of the two VEGF receptors was upregulated in the bone marrow and at the interface between the forming bone and the terminal hypertrophic chondrocytes in the growth plate (Fig. 4I(B,b),II(B,b)). As might be expected from the lack of VEGF expression in Cbfa1 deficient mice, we were unable to detect upregulation of the expression of VEGFR-1 and VEGFR-2 receptors in the perichondrium or the hypertrophic zone (Fig. 4I(C,c),II(C,c)). It is important to emphasize that with the exception of the expression pattern at the interface between the perichondrium and the hypertrophic zone, we did not observe any differences between VEGFR-1

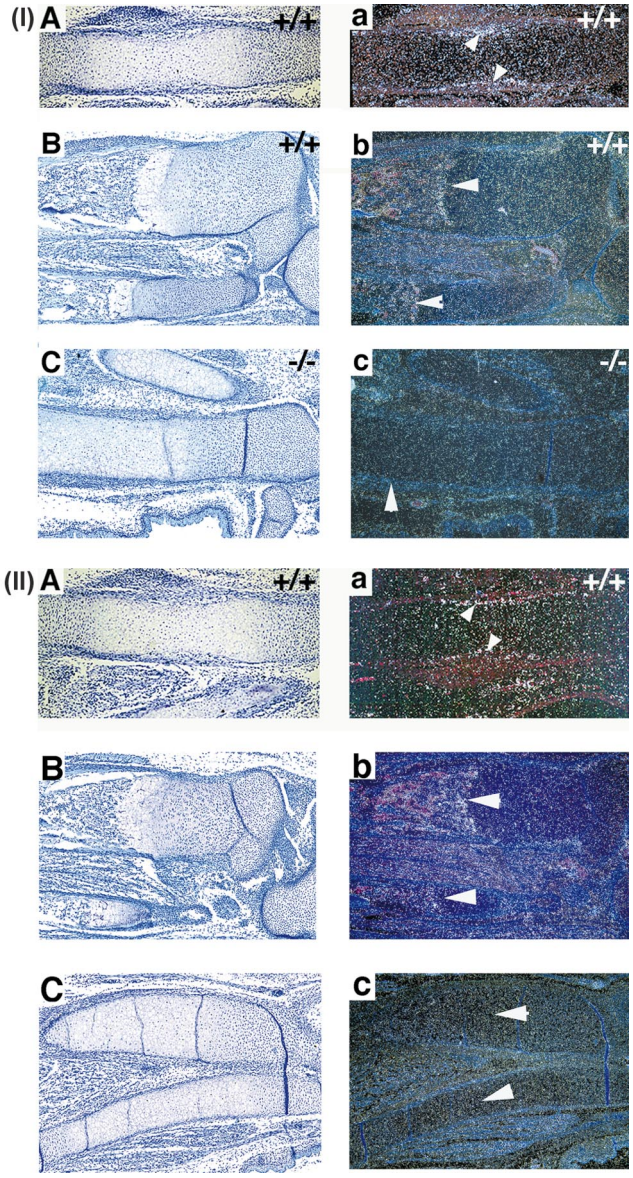

Fig. 4. (I) Expression of VEGFR-2 VEGF receptor in tibias of wild-type and Cbfa1−/− mice. Sections were hybridized with a ^{33}P-labeled VEGFR-2 probe and counterstained with toluidine blue. Panels labeled with capital letters, bright field; panels labeled with small letters, dark field. (A,a) In wild-type mice at E15, VEGFR-2 expression is seen enveloping most of the broad hypertrophic zone in the diaphysis at the perichondrial–cartilage interface (arrowheads). (B,b) Later at E17.5, expression is localized at the interface between the forming bone and the hypertrophic zone of the growth plate (arrowhead). (C,c) In Cbfa1−/− mice at E17.5, no VEGFR-2 expression in the tibia was observed. The broad band of signal seen in (c) is in the surrounding soft tissue. (II) Expression of VEGFR-1 VEGF receptor in tibias of wild-type and Cbfa1−/− mice. Sections were hybridized with a ^{33}P-labeled VEGFR-1 probe and counterstained with toluidine blue. Panels labeled with capital letters, bright field; panels labeled with small letters, dark field. (A,a) Wild-type mouse at E15. (B,b) Wild-type mouse at E17.5. (C,c) Cbfa1 null mouse at E17.5. The signal seen surrounding the bone is in the adjacent soft tissue. The expression pattern of VEGFR-1 in wild-type mice is essentially the same as that of VEGFR-2 at both stages of development. Similar to VEGFR-2, VEGFR-1 is not expressed in the tibias of Cbfa1−/− mice at E17.5.

and VEGFR-2 receptors in wild-type and Cbfa1 deficient mice in the tissues that surround the tibia.

In summary, concomitant with the lack of VEGF expression in the hypertrophic chondrocytes of Cbfa1 deficient mice, there was no upregulation of the expression of VEGF receptors in perichondrial endothelial cells.

2.4. Cbfa1 expression in developing cartilage is progressively upregulated as chondrocytes undergo hypertrophy

In order to better define the role of Cbfa1 in VEGF regulation, we studied the expression of Cbfa1 in developing skeletal elements in wild-type and Cbfa1 heterozygous mice by in situ hybridization and by expression of a LacZ reporter gene that was part of the Cbfa1 targeting construct (Otto et al., 1997) and is transcribed from the Cbfa1 promoter in the knock-out alleles. In situ hybridization in wild-type mice at E15 showed expression of Cbfa1 in chondrocytes, weakly in resting and proliferating chondrocytes, but progressively stronger as chondrocytes matured (Fig. 5(A,a)). At E17.5, this pattern was, in general, maintained, but there was a marked decrease in Cbfa1 expression in the terminal hypertrophic chondrocytes. Very high levels of expression were also observed in the developing trabecular bone (Fig. 5(B,b)). These findings are consistent with previous reports (Inada et al., 1999; Kim et al., 1999). The use of the Cbfa1–LacZ reporter allowed us to observe expression in Cbfa1 null limbs. As shown in Fig. 5(D), strong LacZ staining was seen in newborn Cbfa1 null mice throughout most of the cartilaginous tibia, including the perichondrium, with the exception of the articular regions. As expected, in Cbfa1+/− mice, LacZ staining was less intense than in Cbfa1 −/− animals (Fig. 5(C,D)). The increased expression of Cbfa1 in hypertrophic chondrocytes correlates well with the increased expression of VEGF.

2.5. Cbfa1 induces transcription of VEGF in 3T3 fibroblasts

The results presented above suggested that Cbfa1 is *necessary* for VEGF expression in hypertrophic chondrocytes. To determine whether Cbfa1 is *sufficient* to induce expression of VEGF, we examined the ability of Cbfa1 to induce VEGF expression in cultured cells (Fig. 6). The fact that Cbfa1 overexpression in chondrocytes induces hypertrophy (Enomoto et al., 2000), together with the finding that hypertrophic chondrocytes express VEGF (Carlevaro et al., 2000; Gerber et al., 1999; Haigh et al., 2000), make it difficult to distinguish between Cbfa1 induction of hypertrophy and VEGF regulation by Cbfa1 in chondrocytes. To overcome this problem, we measured VEGF production by NIH3T3 fibroblasts that stably express Cbfa1 following retroviral transduction with pBabe–Cbfa1–3ala. As a control, we used cells that carried the empty vector, pBabe. The cells that carried the empty pBabe vector produced 16 pg VEGF/24 h per 10^4 cells, whereas cells with pBabe–Cbfa1–3ala produced 33 pg VEGF/24 h per 10^4 cells, an increase of 100%. To find out whether this increase is associated with increased levels of VEGF mRNA, we performed quantitative PCR. In cells that

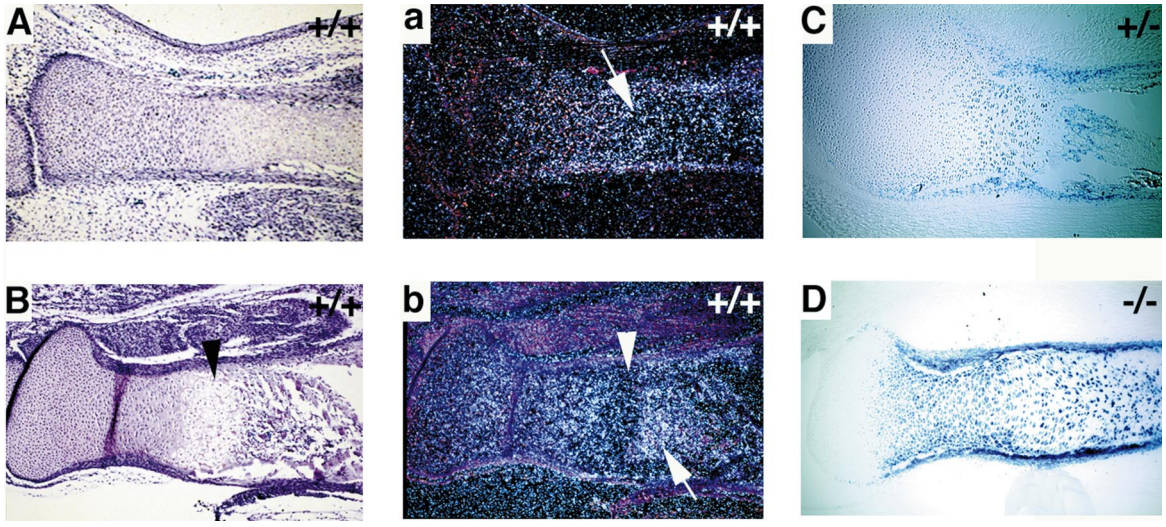

Fig. 5. Expression pattern of Cbfa1. Sections in (A,a) and (B,b) were hybridized with a ^{33}P-labeled Cbfa1 probe and counterstained with toluidine blue. Panels labeled with capital letters, bright field; panels labeled with small letters, dark field. (A,a) Wild-type mouse at E15. Cbfa1 is expressed in the perichondrium/periosteum and within the cartilage primarily within the hypertrophic zones with a progressive increase as chondrocytes mature (arrow). No Cbfa1 expression is seen near or at articular surfaces in cartilage or perichondrium. (B,b) Wild-type mouse at E17.5. Cbfa1 is expressed in the periosteum and in cartilage within the growth plate where it is progressively upregulated as chondrocytes mature, but decreases markedly in terminal hypertrophic chondrocytes (arrowhead). There is also a strong signal in the region of forming bone (arrow). (C) LacZ reporter gene expression in Cbfa1+/− newborn mouse tibia. LacZ staining is present in the periosteum of the shaft, in hypertrophic cartilage of the growth plate and within the trabecular bone, especially subjacent to the growth plate. Note the lack of staining at and near the articular region. (D) LacZ staining of the tibia of a newborn Cbfa1−/− mouse. Staining is more intense than in heterozygous littermates and over a broader area within the tibia.

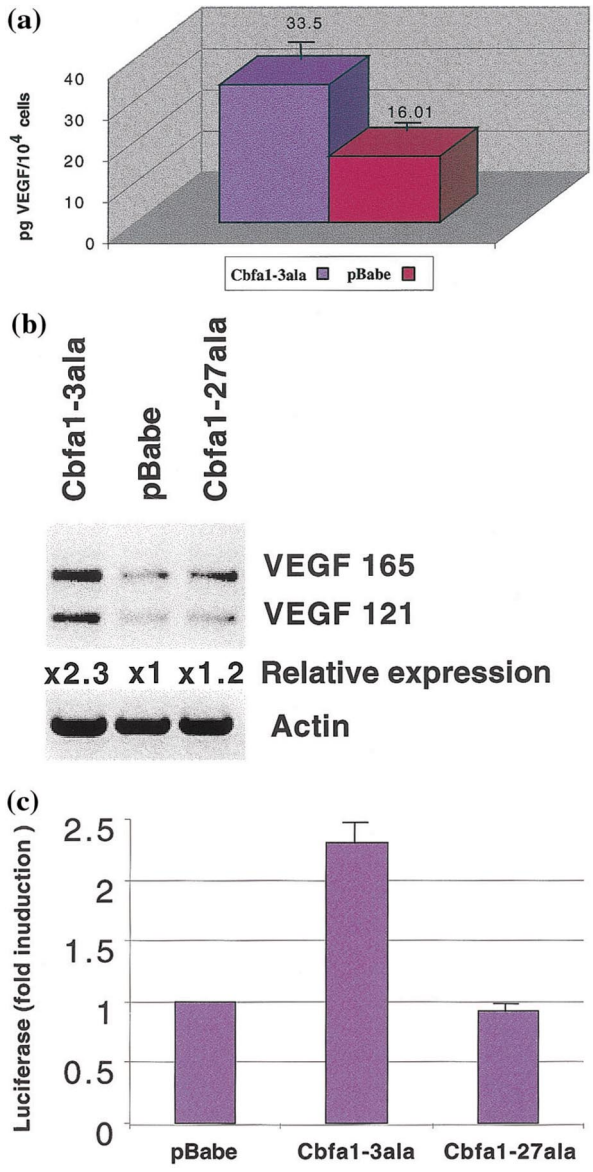

Fig. 6. (A) Induction of VEGF production by ectopic expression of Cbfa1 in 3T3 fibroblasts. The VEGF protein content was determined in conditioned media after 24 h of culture. The results shown are the means of six experiments, each done in duplicate. The purple column shows VEGF production by cells containing the pBabe–Cbfa1–3ala construct and the red column shows VEGF production in cells containing the pBabe control vector. (B) Increase of VEGF mRNA by ectopic expression of Cbfa1 in 3T3 fibroblasts. Real-time PCR was used to determine the relative expression (given as numbers) of VEGF mRNA in cells containing the pBabe–Cbfa1–3ala or the pBabe–Cbfa1–27ala construct compared with levels in cells that contained the pBabe control vector. The PCR products were separated by electrophoresis through a 1% agarose gel. The ethidium bromide stained gel showed the presence of both VEGF120 and VEGF164 isoforms in relative quantities commensurate with the results of the real-time PCR. PCR with primers for β-actin was used as an internal control. (C) Ectopic expression of Cbfa1 in 3T3 fibroblasts induces transcription from the VEGF promoter. 3T3 fibroblasts cells carrying the retroviral construct pBabe–Cbfa1–3ala, pBabe–Cbfa1–27ala or the empty vector pBabe were transiently transfected with 1 μg of the VEGF–PR–LUC plasmid DNA and 1 μg of pPGKβGal plasmid DNA as an internal control. The relative luciferase activities represent average values of three independent experiments.

were infected by pBabe–Cbfa1–3ala, the steady-state level of VEGF mRNA was two-fold higher (for both the VEGF-120 and VEGF-164 isoforms) than in cells that were infected by the pBabe empty vector or by pBabe–Cbfa1–27ala, an inactive form of Cbfa1 (Thirunavukkarasu et al., 1998). The ability of Cbfa1 to increase the steady-state level of VEGF mRNA is in line with the increment in the production of VEGF protein by the cells. To determine whether or not the increased steady-state level of VEGF mRNA was a consequence of increased transcription, we transiently transfected the three different pBabe-containing NIH3T3 lines with a construct that contains a 3.2 kb human VEGF promoter region fused to luciferase cDNA (VEGF–PR–LUC). These experiments demonstrated a two-fold induction of luciferase activity relative to the two control vectors, pBabe and pBabe–Cbfa1–27ala. We conclude, therefore, that Cbfa1 is capable of inducing VEGF production by regulating its transcription.

3. Discussion

Little is known about tissue specific regulation of VEGF during embryonic development. VEGF plays a major role in the invasion of hypertrophic cartilage by blood vessels during endochondral bone formation, thus providing an attractive biological context in which to analyze such regulation. In this study, we present evidence for a link between the transcription factor Cbfa1 and regulation of VEGF. In wild-type mice, VEGF gene expression is upregulated in hypertrophic chondrocytes; in Cbfa1 deficient mice, this upregulation does not occur. Furthermore, the induction of both VEGF receptors, VEGFR-1 and VEGFR-2, in perichondrial endothelial cells does not occur in Cbfa1 deficient mice. The lack of VEGF expression in the growth plates of Cbfa1 null mice is the first example of tissue specific loss of VEGF expression as a result of targeting a single gene. Finally, over-expression of Cbfa1 in cultured cells induces the transcription and production of VEGF.

3.1. Cbfa1 is involved in the regulation of angiogenesis in the forming bone

Cbfa1 plays several major roles during bone development. One function is that of a regulator of osteoblast differentiation (Ducy et al., 1997); in Cbfa1 deficient mice, the skeleton is composed entirely of cartilage and no osteoblasts are formed (Komori et al., 1997; Otto et al., 1997). Other functions include the regulation of chondrocyte maturation (Inada et al., 1999; Kim et al., 1999). During skeletal development in wild-type mice, angiogenesis is coupled spatially and temporally to chondrocyte hypertrophy, but in Cbfa1 deficient mice, there is no vascularization of cartilage (Fig. 1II(B)). This lack of angiogenesis is not simply a consequence of a lack of hypertrophy in Cbfa1 deficient mice, because in distal long bones, such as the tibia, fibula, radius, and ulna, where chondrocytes do differentiate to hypertro-

phy (Fig 1I(C,c)), no blood vessel invasion of the cartilage can be demonstrated with anti-CD-31 staining, although there is an abundance of blood vessels in the perichondrium (Fig. 1II(B,C)). The hypertrophic cartilage in the tibia of wild-type and Cbfa1 deficient mice appears identical by histological criteria, and the extracellular matrix calcifies in the same way. On a molecular level, the expression sequence of a number of chondrocyte markers, such as Indian hedgehog, collagen X, and bone morphogenetic protein 6, during hypertrophy in the tibia of Cbfa1 deficient mice follows that of wild-type animals (Inada et al., 1999; Kim et al., 1999). Also, the genes that are required for the proper expression of Cbfa1 in chondrocytes are apparently not affected by the loss of Cbfa1 protein, since staining for the LacZ reporter transcribed from the Cbfa1 promoter in the Cbfa1 null mice showed staining of chondrocytes (Fig. 5(C,D)) similar to that seen by in situ hybridization for Cbfa1 in wild-type mice.

The extensive hypertrophic zone in the tibia of Cbfa1 deficient mice may be the result of a lack of blood vessel-associated invasion of osteoclasts. In fact, the expansion of the hypertrophic zone seen in mutant animals resembles cartilage phenotypes that are observed when the blood supply to the forming bone is altered. For example, expansion of the hypertrophic zone is observed after partial interruption of the blood supply to the growth plate in rabbits (Trueta and Amato, 1960). Moreover, mice lacking genes that play a role in osteoclast invasion and vascularization of cartilage, such as MMP-9 and VEGF, have similar phenotypes (Engsig et al., 2000; Haigh et al., 2000; Vu et al., 1998). The lack of expression of other genes in terminal hypertrophic chondrocytes of Cbfa1 deficient mice could also contribute to the phenotype. In fact, several genes whose transcription is regulated directly by Cbfa1 show a reduced expression in Cbfa1 deficient mice (Inada et al., 1999; Kim et al., 1999). These genes include osteopontin, osteocalcin, bone sialoprotein and MMP-13 (Javed et al., 2001; Jimenez et al., 1999; Sato et al., 1998; Schinke and Karsenty, 1999) and their absence may contribute to the lack of angiogenesis and removal of hypertrophic matrix.

3.2. VEGF expression is part of a tissue specific program that is regulated by Cbfa1

Our results indicate that Cbfa1 is involved in regulating cartilage vascularization by controlling the expression of VEGF. Upregulation of VEGF expression in hypertrophic chondrocytes and the concomitant upregulation of the VEGF receptors in the perichondrium/periosteum seen in wild-type mice was not seen in the Cbfa1 deficient animal. The increase in the receptor signal can be explained by VEGF-induced recruitment of endothelial cells to the interface between the forming bone and the hypertrophic cartilage and to an increase in the expression of the receptors by these cells. The strongest signal was seen at the cartilage–bone junction; this site includes osteoblasts and hematopoietic precursors, it is possible that these cells contribute to the observed increase in receptor signal (Deckers et al., 2000).

Over-expression of Cbfa1 in NIH3T3 fibroblasts increased both the transcription and production of VEGF in these cells by 100% relative to the control vector. The amount of this increase is commensurate with the extent of VEGF upregulation seen with other stimuli of VEGF expression, such as hypoxia, insulin and cobalt chloride (Liu et al., 1995; Zelzer et al., 1998). In a recent study, Takeda et al. (G. Karsenty, personal communication) found that restoring the expression of Cbfa1 in non-hypertrophic chondrocytes induces chondrocyte hypertrophy, VEGF expression and vascularization of cartilage in Cbfa1 deficient mice. Taken together, these data strongly suggest that Cbfa1 regulates VEGF gene expression during endochondral bone formation in a tissue specific manner. Since Cbfa1 is expressed in both osteoblasts and chondrocytes, it is reasonable to assume that Cbfa1 is involved in regulating genes which these cells express in common. Since VEGF is expressed by both cell types (Deckers et al., 2000), it may be that Cbfa1 regulates VEGF expression in osteoblasts as well as in chondrocytes. Although Cbfa1 expression in NIH3T3 cells is sufficient to upregulate the transcription and production of VEGF, it is possible that other genes, in addition to Cbfa1, are needed for the expression of VEGF in hypertrophic chondrocytes. This could explain why upregulation of VEGF expression occurs only in the hypertrophic cells in the growth plate, whereas Cbfa1 is also expressed by proliferating, non-hypertrophic chondrocytes. Another possibility is that Cbfa1 activity is repressed in proliferating chondrocytes. Little is known about the factors that regulate Cbfa1 expression and activity in vivo, but bone morphogenetic proteins (BMPs) and signaling through BMP receptors appear to be involved (for review, see Yamaguchi et al., 2000). Also, interactions with members of the groucho-family and posttranslational modification (phosphorylation) are likely to modulate the activity of Cbfa1 (McLarren et al., 2000; Selvamurugan et al., 2000).

VEGF is a central molecule in development. Although hypoxia was shown in previous studies to play an important role in its regulation, we demonstrate here that a transcription factor, Cbfa1, regulating bone organogenesis controls VEGF expression in a tissue specific manner. This ensures coupling of organogenesis with angiogenesis. Tissue specific expression of VEGF in other organs during development could well be controlled in a similar fashion by other transcription factors.

4. Experimental procedures

4.1. In situ hybridization

In situ hybridization was carried out on paraffin sections with ^{33}P-labeled RNA essentially as described by Hartmann and Tabin (Hartmann and Tabin, 2000). Slides were hybridized at 60°C in a humidified chamber. The VEGF probe

was a gift from B. Cohen (Weizmann Institute, Israel). The collagen X probe consisted of a 0.65 kb cDNA fragment encoding part of the carboxyl domain NC1 and the 3′-UTR. The VEGFR-1 probe consisted of a 1.6 kb cDNA fragment (Finnerty et al., 1993) and the VEGFR-2 probe was a 1 kb cDNA fragment (Quinn et al., 1993). The Cbfa1 probe consisted of a 0.73 kb fragment covering nucleotides 680–1412 in the GenBank entry AF010284.

4.2. Generation of retroviral constructs

C-terminal influenza virus hemagglutinin (FLAG)-tagged constructs of the mouse Cbfa1 cDNA were generated using a PCR based approach. As templates, we used Cbfa1 mutants, in which 15 of 18 alanines in the QA domain have been deleted (Cbfa1–3ala) or nine alanines have been added (Cbfa1–27ala). Cbfa1–3ala is reported to have wild-type transactivation activity, whereas Cbfa1–27ala is inactive (Thirunavukkarasu et al., 1998); both were kindly provided by Dr P. Ducy. The retroviral constructs were generated by subcloning the *Eco*RI–*Eco*NI and *Eco*NI–*Xho*I fragments of both Cbfa1 mutants into the *Eco*RI–*Xho*I site of the vector pBabe–puro (Morgenstern and Land, 1990). The retrovirus was amplified and purified in the amphotropic packaging cell line Phoenix 293 (courtesy of Dr Gary Nolan), according to the method of Pear et al. (Pear et al., 1993), with modifications (Nolan website: http://www.stanford.edu/group/nolan/NL-helper.html). Briefly, Phoenix 293 cells were grown in DME containing 10% fetal calf serum (FCS). At 50% confluence, cells in 15 cm plates were transfected with the expression constructs, using FuGene (Roche Molecular Biochemicals, IN). Within 12–16 h, 10 ml fresh medium was added. This medium, containing viral particles, was harvested and flash-frozen in liquid nitrogen. For transduction, NIH3T3 cells on 10 cm plates were grown to 50% confluence in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS. The medium was removed and replaced with 5 ml viral supernatant for 4 h. At this time, fresh medium was added to a volume of 10 ml, and cultures were allowed to incubate overnight before removal of the viral supernatant and addition of fresh medium. After 24 h, fresh medium containing 2 μg/ml puromycin was added. The medium was changed daily or as required for 1 week, at which time selection was complete, and expression of the pBabe–Cbfa1–3ala–FLAG and pBabe–Cbfa1–27ala–FLAG constructs was confirmed by Western blot analysis.

4.3. VEGF protein measurement

3T3 fibroblasts cells carrying either the retroviral construct pBabe–Cbfa1–3ala or the empty vector were plated at a density of 5×10^5 cells/well in 24-well plates and cultured for 24 h in DME containing 10% FCS. Medium from each well was collected and VEGF was measured by a specific mouse VEGF immunoassay kit (Quantikine M, R&D Systems). The culture medium did not contain detectable levels of VEGF.

4.4. Quantitative RT-PCR

Total RNA (1 μg) was reverse transcribed for 50 min at 42°C in 20 μl reaction mixture containing RT-buffer (50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3mM $MgCl_2$ and 10 mM DTT), 0.5 mM of each dNTP, 100 μM of random oligodeoxynucleotide hexamers, and 50 U of reverse transcriptase (Moloney murine leukemia virus Super Script II H-Gibco BRL). Components of the SYBR Green Core PCR Reagent Kit (PE ABI, Foster City, CA) were used for PCR amplification of VEGF and *β-actin* from 2 μl of a 1:10 dilution of first strand cDNA on an iCycler iQ real-time PCR system (BioRad, Hercules, CA). The cycling parameters were: 50°C for 3 min, 94°C for 10 min, followed by 50 cycles of 94°C for 30 s, 63°C for 30 s, and 72°C for 30 s. In each experiment, three identical 20 μl PCR reactions of VEGF and the control gene (*β-actin*) were run. The primers for VEGF were forward: 5′-GGGTGCACTGGACCCTGGGTTTAC-3′ and reverse: 5′-CCTGGCTCACCGCCTTGGCTTGTC-3′.

Relative expression levels between the different cell lines were calculated separately for each experiment. Threshold cycles were determined using the default threshold levels (ten-fold standard deviation from cycle 2 to 10), and the average threshold cycle for VEGF was normalized for amplification of *β-actin*, assuming an amplification efficiency of 2.0 in all reactions.

4.5. Transient transfections

3T3 fibroblast cells carrying the retroviral construct pBabe–Cbfa1–3ala, pBabe–Cbfa1–27ala or the empty vector were plated at a density of 1×10^5 cells/well in six-well plates and cultured overnight in DME containing 10% FCS. Transient transfections of the 3.2 kb hVEGF promoter-containing construct VEGF–PR–LUC by Lipofectamine plus reagent (Life Technologies) were carried out according to the manufacturer's protocol. pPGKβGal were used for normalization of transfection efficiency. The transfected cells were harvested 24–48 h after transfection and assayed for luciferase activity using the dual-luciferase reporter assay system (Promega). The luciferase activities were normalized to the activity obtained with the pBabe control vector.

4.6. Immunohistochemistry

For CD31 immunohistochemistry, embryos were fixed in 4% paraformaldehyde at 4°C for 12 h, followed by 20% sucrose at 4°C for 12 h. Tissues were embedded in OCT (Tissue-Tek) and 7 μm sections were made. The immunohistochemistry on sections was performed using an automated staining system (BiogenexOptimax Plus). Sections were incubated with 0.1% trypsin in water for 10 min,

then incubated for 2 h with 0.6 mg/ml monoclonal rat anti-mouse CD31 (PharMingen). After washing, sections were incubated with biotinylated anti-rat IgG for 30 min. For detection, a supersensitive detection kit (BioGenex) was utilized within a 20 min incubation.

4.7. LacZ staining

LacZ staining was performed on cryosections. Lower extremities were fixed in a solution containing 0.2% paraformaldehyde, 0.1 M PIPES, 2 mM $MgCl_2$ and 5 mM EGTA (0.2% para/pipes) for 10 h at 4°C on a shaker. The tissue was then infiltrated overnight at 4°C with 30% sucrose in phosphate-buffered saline (PBS) containing 2 mM $MgCl_2$, embedded in Tissue-Tek® OCT compound which was frozen in ethanol and dry ice and stored at −80°C. Sections of 8 μm were cut on a cryostat, mounted on Superfrost Plus® slides and stored at room temperature. Just prior to staining, the sections were further fixed in 2% para/pipes for 10 min at 4°C and then rinsed for 10 min at 4°C in a solution containing 0.1 M sodium phosphate (pH 7.4), 0.1% sodium deoxycholate, 2 mM $MgCl_2$ and 0.2% NP-40. The slides were then placed in a solution containing 1 mg/ml X-Gal (Sigma), 5 mM K-ferrocyanide and 5 mM K-ferricyanide and incubated for 16 h at 37°C. They were washed successively in PBS/2 mM $MgCl_2$, 2% para/pipes, and PBS/2 mM $MgCl_2$ again, each for 5 min at room temperature. A coverslip was then applied with gelvatol mounting medium.

Acknowledgements

The authors are grateful to Dr M. Owen for providing breeding pairs of the Cbfa1 deficient mice, to Dr B. Cohen for providing the VEGF probe, and to Dr E. Tischer for providing the human VEGF promoter fragment. The authors wish to thank W. Wang, D. Rieger, R. Mamluk, and M. Klagsbrun for helpful review of the manuscript, S. Plotkina for excellent technical help, and Y. Pittel for patience in editing the manuscript. Special thanks to all members of the Olsen laboratory for continuous advice and suggestions and to R. Schweitzer for his endless good ideas. This work was supported by EMBO (to E. Zelzer) and NIH grants AR36819 and AR36820 (to B.R. Olsen).

References

Barleon, B., Siemeister, G., Martiny-Baron, G., Weindel, K., Herzog, C., Marme, D., 1997. Vascular endothelial growth factor up-regulates its receptor fms-like tyrosine kinase 1 (FLT-1) and a soluble variant of FLT-1 in human vascular endothelial cells. Cancer Res. 57, 5421–5425.

Carlevaro, M.F., Cermelli, S., Cancedda, R., Descalzi Cancedda, F., 2000. Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation. J. Cell Sci. 113, 59–69.

Carmeliet, P., Collen, D., 1999. Role of vascular endothelial growth factor and vascular endothelial growth factor receptors in vascular development. Curr. Top. Microbiol. Immunol. 237, 133–158.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W., Nagy, A., 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380, 435–439.

Cohen, T., Nahari, D., Cerem, L.W., Neufeld, G., Levi, B.Z., 1996. Interleukin 6 induces the expression of vascular endothelial growth factor. J. Biol. Chem. 271, 736–741.

Deckers, M.M., Karperien, M., van der Bent, C., Yamashita, T., Papapoulos, S.E., Lowik, C.W., 2000. Expression of vascular endothelial growth factors and their receptors during osteoblast differentiation. Endocrinology 141, 1667–1674.

Ducy, P., Zhang, R., Geoffroy, V., Ridall, A.L., Karsenty, G., 1997. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 89, 747–754.

Engsig, M.T., Chen, Q.J., Vu, T.H., Pedersen, A.C., Therkidsen, B., Lund, L.R., Henriksen, K., Lenhard, T., Foged, N.T., Werb, Z., Delaisse, J.M., 2000. Matrix metalloproteinase 9 and vascular endothelial growth factor are essential for osteoclast recruitment into developing long bones. J. Cell Biol. 151, 879–890.

Enomoto, H., Enomoto-Iwamoto, M., Iwamoto, M., Nomura, S., Himeno, M., Kitamura, Y., Kishimoto, T., Komori, T., 2000. Cbfa1 is a positive regulatory factor in chondrocyte maturation. J. Biol. Chem. 275, 8695–8702.

Eriksson, U., Alitalo, K., 1999. Structure, expression and receptor-binding properties of novel vascular endothelial growth factors. Curr. Top. Microbiol. Immunol. 237, 41–57.

Ferrara, N., 1999. Molecular and biological properties of vascular endothelial growth factor. J. Mol. Med. 77, 527–543.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K.S., Powell-Braxton, L., Hillan, K.J., Moore, M.W., 1996. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380, 439–442.

Finnerty, H., Kelleher, K., Morris, G.E., Bean, K., Merberg, D.M., Kriz, R., Morris, J.C., Sookdeo, H., Turner, K.J., Wood, C.R., 1993. Molecular cloning of murine FLT and FLT4. Oncogene 8, 2293–2298.

Fong, G.H., Rossant, J., Gertsenstein, M., Breitman, M.L., 1995. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 376, 66–70.

Gerber, H.P., Vu, T.H., Ryan, A.M., Kowalski, J., Werb, Z., Ferrara, N., 1999. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat. Med. 5, 623–628.

Goad, D.L., Rubin, J., Wang, H., Tashjian, A.H.J., Patterson, C., 1996. Enhanced expression of vascular endothelial growth factor in human SaOS-2 osteoblast-like cells and murine osteoblasts induced by insulin-like growth factor I. Endocrinology 137, 2262–2268.

Goldman, C.K., Kim, J., Wong, W.L., King, V., Brock, T., Gillespie, G.Y., 1993. Epidermal growth factor stimulates vascular endothelial growth factor production by human malignant glioma cells: a model of glioblastoma multiforme pathophysiology. Mol. Biol. Cell 4, 121–133.

Haigh, J.J., Gerber, H.P., Ferrara, N., Wagner, E.F., 2000. Conditional inactivation of VEGF-A in areas of collagen2a1 expression results in embryonic lethality in the heterozygous state. Development 127, 1445–1453.

Hartmann, C., Tabin, C.J., 2000. Dual roles of wnt signaling during chondrogenesis in the chicken limb. Development 127, 3141–3159.

Inada, M., Yasui, T., Nomura, S., Miyake, S., Deguchi, K., Himeno, M., Sato, M., Yamagiwa, H., Kimura, T., Yasui, N., Ochi, T., Endo, N., Kitamura, Y., Kishimoto, T., Komori, T., 1999. Maturational disturbance of chondrocytes in Cbfa1-deficient mice. Dev. Dyn. 214, 279–290.

Iyama, K.-I., Ninomiya, Y., Olsen, B.R., Linsenmayer, T.F., Trelstad, R.L., Hayashi, M., 1991. Spatio-temporal pattern of type X collagen gene expression and collagen deposition in embryonic chick vertebrae undergoing endochondral ossification. Anat. Rec. 229, 462–472.

Javed, A., Barnes, G.L., Jasanya, B.O., Stein, J.L., Gerstenfeld, L., Lian,

J.B., Stein, G.S., 2001. runt homology domain transcription factors (runx, cbfa, and aml) mediate repression of the bone sialoprotein promoter: evidence for promoter context-dependent activity of cbfa proteins. Mol. Cell. Biol. 21, 2891–2905.

Jimenez, M.J., Balbin, M., Lopez, J.M., Alvarez, J., Komori, T., Lopez-Otin, C., 1999. Collagenase 3 is a target of Cbfa1, a transcription factor of the runt gene family involved in bone formation. Mol. Cell. Biol. 19, 4431–4442.

Jingushi, S., Scully, S.P., Joyce, M.E., Sugioka, Y., Bolander, M.E., 1995. Transforming growth factor-beta 1 and fibroblast growth factors in rat growth plate. J. Orthop. Res. 13, 761–768.

Kaipainen, A., Korhonen, J., Mustonen, T., van Hinsbergh, V.W., Fang, G.H., Dumont, D., Breitman, M., Alitalo, K., 1995. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc. Natl. Acad. Sci. USA 92, 3566–3570.

Karsenty, G., 2000. Role of Cbfa1 in osteoblast differentiation and function. Semin. Cell Dev. Biol. 11, 343–346.

Kim, I.S., Otto, F., Zabel, B., Mundlos, S., 1999. Regulation of chondrocyte differentiation by Cbfa1. Mech. Dev. 80, 159–170.

Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R.T., Gao, Y.H., Inada, M., Sato, M., Okamoto, R., Kitamura, Y., Yoshiki, S., Kishimoto, T., 1997. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89, 755–764.

Kotch, L.E., Iyer, N.V., Laughner, E., Semenza, G.L., 1999. Defective vascularization of HIF-1alpha-null embryos is not associated with VEGF deficiency but with mesenchymal cell death. Dev. Biol. 209, 254–267.

Li, J., Perrella, M.A., Tsai, J.C., Yet, S.F., Hsieh, C.M., Yoshizumi, M., Patterson, C., Endege, W.O., Zhou, F., Lee, M.E., 1995. Induction of vascular endothelial growth factor gene expression by interleukin-1 beta in rat aortic smooth muscle cells. J. Biol. Chem. 270, 308–312.

Liu, Y., Cox, S.R., Morita, T., Kourembanas, S., 1995. Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5′ enhancer. Circ. Res. 77, 638–643.

McLarren, K.W., Lo, R., Grbavec, D., Thirunavukkarasu, K., Karsenty, G., Stifani, S., 2000. The mammalian basic helix loop helix protein HES-1 binds to and modulates the transactivating function of the runt-related factor Cbfa1. J. Biol. Chem. 275, 530–538.

Morgenstern, J.P., Land, H., 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. 18, 3587–3596.

Neufeld, G., Cohen, T., Gengrinovitch, S., Poltorak, Z., 1999. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J. 13, 9–22.

Ninomiya, Y., Gordon, M., van der Rest, M., Schmid, T., Linsenmayer, T., Olsen, B.R., 1986. The developmentally regulated type X collagen gene contains a long open reading frame without introns. J. Biol. Chem. 261, 5041–5050.

Olsen, B.R., Reginato, A.M., Wang, W., 2000. Bone development. Annu. Rev. Cell Dev. 16, 191–220.

Otto, F., Thornell, A.P., Crompton, T., Denzel, A., Gilmour, K.C., Rosewell, I.R., Stamp, G.W., Beddington, R.S., Mundlos, S., Olsen, B.R., Selby, P.B., Owen, M.J., 1997. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89, 765–771.

Pear, W.S., Nolan, G.P., Scott, M.L., Baltimore, D., 1993. Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl. Acad. Sci. USA 90, 8392–8396.

Quinn, T.P., Peters, K.G., De Vries, C., Ferrara, N., Williams, L.T., 1993. Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. Proc. Natl. Acad. Sci. USA 90, 7533–7537.

Sato, M., Morii, E., Komori, T., Kawahata, H., Sugimoto, M., Terai, K., Shimizu, H., Yasui, T., Ogihara, H., Yasui, N., Ochi, T., Kitamura, Y., Ito, Y., Nomura, S., 1998. Transcriptional regulation of osteopontin gene in vivo by PEBP2alphaA/CBFA1 and ETS1 in the skeletal tissues. Oncogene 17, 1517–1525.

Schinke, T., Karsenty, G., 1999. Characterization of Osf1, an osteoblast-specific transcription factor binding to a critical cis-acting element in the mouse Osteocalcin promoters. J. Biol. Chem. 274, 30182–30189.

Selvamurugan, N., Pulumati, M.R., Tyson, D.R., Partridge, N.C., 2000. Parathyroid hormone regulation of the rat collagenase-3 promoter by protein kinase A-dependent transactivation of core binding factor alpha1. J. Biol. Chem. 275, 5037–5042.

Semenza, G.L., Wang, G.L., 1992. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol. Cell. Biol. 12, 5447–5454.

Shalaby, F., Rossant, J., Yamaguchi, T.P., Gertsenstein, M., Wu, X.F., Breitman, M.L., Schuh, A.C., 1995. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 376, 62–66.

Shalaby, F., Ho, J., Stanford, W.L., Fischer, K.D., Schuh, A.C., Schwartz, L., Bernstein, A., Rossant, J., 1997. A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. Cell 89, 981–990.

Shen, B.Q., Lee, D.Y., Gerber, H.P., Keyt, B.A., Ferrara, N., Zioncheck, T.F., 1998. Homologous up-regulation of KDR/Flk-1 receptor expression by vascular endothelial growth factor in vitro. J. Biol. Chem. 273, 29979–29985.

Shinar, D.M., Endo, N., Halperin, D., Rodan, G.A., Weinreb, M., 1993. Differential expression of insulin-like growth factor-I (IGF-I) and IGF-II messenger ribonucleic acid in growing rat bone. Endocrinology 132, 1158–1167.

Shweiki, D., Itin, A., Soffer, D., Keshet, E., 1992. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359, 843–845.

Thirunavukkarasu, K., Mahajan, M., McLarren, K.W., Stifani, S., Karsenty, G., 1998. Two domains unique to osteoblast-specific transcription factor Osf2/Cbfa1 contribute to its transactivation function and its inability to heterodimerize with Cbfbeta. Mol. Cell. Biol. 18, 4197–4208.

Trueta, J., Amato, V.P., 1960. The vascular contribution of osteogenesis. III. Changes in the growth cartilage caused by experimentally induced ischaemia. J. Bone Joint Surg. 42B, 571–587.

Vu, T.H., Shipley, J.M., Bergers, G., Berger, J.E., Helms, J.A., Hanahan, D., Shapiro, S.D., Senior, R.M., Werb, Z., 1998. MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. Cell 93, 411–422.

Yamaguchi, A., Komori, T., Suda, T., 2000. Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1. Endocr. Rev. 21, 393–411.

Zelzer, E., Levy, Y., Kahana, C., Shilo, B.Z., Rubinstein, M., Cohen, B., 1998. Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1alpha/ARNT. EMBO J. 17, 5085–5094.