VEGFA is necessary for chondrocyte survival during bone development

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Summary

To directly examine the role of vascular endothelial growth factor (VEGFA) in cartilage development, we conditionally knocked out Vegfa in chondrocytes, using the Col2a1 promoter to drive expression of Cre recombinase. Our study of Vegfa conditional knockout (CKO) mice provides new in-vivo evidence for two important functions of VEGFA in bone formation. First, VEGFA plays a significant role in both early and late stages of cartilage vascularization, since Vegfa CKO mice showed delayed invasion of blood vessels into primary ossification centers and delayed removal of terminal hypertrophic chondrocytes. Second, VEGFA is crucial for chondrocyte survival, since massive cell death was seen in joint and epiphyseal regions of Vegfa CKO endochondral bones. Chondrocytes in these regions were found to upregulate expression of Vegfa in wild-type mice at the time when massive cell death occurred in the Vegfa CKO mice. The expression of the VEGFA receptors Npr1 and Npr2 in epiphyseal chondrocytes and lack of blood vessel reduction in the vicinity of the cartilaginous elements in the Vegfa CKO mice raise the possibility that the observed cell death is the result of a direct involvement of VEGFA in chondrocyte survival. Interestingly, the extensive cell death seen in Vegfa CKO null bones had a striking similarity to the cell death phenotype observed when hypoxia-inducible factor 1α (Hif1α) expression was abolished in developing cartilage. This similarity of cell death phenotypes and the deficient VEGFA production in Hif1α null epiphyseal chondrocytes demonstrate that HIF1α and VEGFA are components of a key pathway to support chondrocyte survival during embryonic bone development.

Key words: Conditional knockout mice, VEGFA, Chondrocyte survival, Bone development, Angiogenesis, HIF1, HIF1α, VEGF

Introduction

The vertebral skeleton is formed by cells that originate from the cranial neural crest, somites, and the lateral plate mesoderm. Bones in the cranial vault, jaws, and part of the clavicle form by intramembranous ossification, a process in which mesenchymal cells differentiate into osteoblasts (Hall and Miyake, 1992). The rest of the skeleton develops by a process known as endochondral ossification. During endochondral ossification, mesenchymal cells differentiate into chondrocytes, which then proliferate and produce cartilage models (templates, anlagen) of the future bones. As development proceeds, chondrocytes in the center of each of the cartilage templates cease to proliferate and the post-mitotic cells differentiate to hypertrophy. The differentiation of chondrocytes to hypertrophy is followed by 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 in the so-called primary ossification centers (Karsenty, 1999; Olsen et al., 2000).

Vascular endothelial growth factor A (VEGFA) is an important regulator of angiogenesis during endochondral ossification. Inhibition of VEGFA by administration of soluble chimeric VEGFA receptor protein to 24-day-old mice inhibited blood vessel invasion into the hypertrophic zone of long bone growth plates and resulted in impaired trabecular bone formation and the expansion of the hypertrophic zone (Gerber et al., 1999). Further support for the role of VEGFA in angiogenesis of hypertrophic cartilage came from studies of mice expressing only the VEGFA120 isoform of VEGFA (Maes et al., 2002; Zelzer et al., 2002).

VEGFA120 is one of three isoforms of VEGFA in the mouse (Ferrara et al., 1992; Shima et al., 1996). VEGFA120 does not bind heparan sulfate, while the other two isoforms, VEGFA164 and VEGFA188, possess one or two heparin-binding domains, respectively, allowing interactions with heparan sulfate (Ferrara and Davis-Smyth, 1997; Park et al., 1993). Unlike heterozygous Vegfa null mice, mice that express only the 120 isoform survive through embryonic development (Carmeliet et al., 1999). Studies of blood vessel invasion into the primary ossification centers in VEGFA120 null mice demonstrated a delay in vessel invasion and alterations in the extent of expression of...
cartilage differentiation markers at E14.5, indicating a role for VEGFA in cartilage angiogenesis and maturation (Maes et al., 2002; Zelzer et al., 2002). The skeletons of VEGFA120 mice showed decreased mineralization and a reduction in the expression of osteoblastic markers in membranous and endochondral bone. This suggests that VEGFA has a direct effect on the activity of osteoblasts (Zelzer et al., 2002). There is also evidence from in-vitro experiments that VEGFA may regulate bone formation through a direct effect on osteoblasts (Deckers et al., 2000; Midy and Plouet, 1994).

Thus, VEGFA appears to have several functions during bone formation. In this study, we report for the first time that VEGFA is critical for chondrocyte survival. We demonstrate that VEGFA, in addition to its upregulated expression in hypertrophic chondrocytes of long bone growth plates, is expressed at moderate levels in epiphyseal chondrocytes, and that these chondrocytes undergo massive cell death in the absence of VEGFA. Based on the striking similarity in the cartilage phenotypes resulting from inactivation of either Vegf or hypoxia-inducible factor 1α (Hif1α) in chondrocytes, we conclude that the chondrocyte survival function of VEGFA is controlled by HIF1α.

Materials and methods

Animals

The generation of floxed-Vegfa (Gerber et al., 1999), floxed-Hif1α (Schipani et al., 2001), Col2-Cre (Schipani et al., 2001), and Prx1-Cre (Logan et al., 2002) mice have been described previously. In all timed pregnancies, plug date was defined as E0.5. For harvesting of embryos, timed-pregnant female mice were killed by exposure to CO2. The gravid uterus was dissected out and suspended in a bath of cold PBS and the embryos were delivered after amnionectomy and removal of the placenta. Genomic DNA, isolated using standard protocols from portions of the embryos (the tail in E15.5 and older embryos), was used for genotyping.

Skeletal preparations

Cartilage and bones in whole mouse embryos (E18.5) were visualized after staining with Alcian Blue and Alizarin Red S (Sigma) and clarification of soft tissue with potassium hydroxide (McLeod, 1980).

Histology and immunohistochemistry

For histological analysis, embryonic limbs and heads were fixed in 4% paraformaldehyde and embedded in paraffin for sectioning using standard protocols. Sections of 7 μm thickness were stained with hematoxylin and eosin (H & E). For CD31 immunohistochemistry, embryos were fixed in 4% paraformaldehyde, followed by 20% sucrose infiltration. Tissues were embedded in OCT (Tissue-Tek®) and 7 μm cryostat sections were made. Sections were incubated with monoclonal rat anti-mouse CD31 (BD PharMingen). Sections were incubated with biotinylated anti-rat IgG (Vector Laboratories), and an ABC kit (Vector Laboratories) was used for detection.

In situ hybridization and TUNEL assay

In situ hybridization was carried out on paraffin sections with 33P-labeled anti-sense RNA essentially as described (Hartmann and Tabin, 2000; Zelzer et al., 2002). For TUNEL assay, paraffin sections were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. TUNEL assay was performed using a Roche In situ Cell Death Detection kit (Roche) according to the manufacturer’s recommendations.

Culture of primary epiphyseal chondrocytes

Epiphyseal chondrocytes were isolated from the knee joint region of newborn mice. Skin, muscles and soft tissue were removed. DMEM containing 0.5 mg/ml hyaluronidase (Sigma H-3506) was added and the tissue was digested for 30 minutes at 37°C on a rotating shaker. The medium was then replaced by medium containing 1 mg/ml trypsin (Sigma T-1426) and incubation continued for 30 minutes at 37°C on a rotating shaker. The trypsin containing medium was removed and cartilage was incubated twice with medium containing 1 mg/ml bacterial collagenase (Sigma C-9263). Medium from the first incubation was discarded after 20 minutes and then chondrocytes were isolated by collagenase digestion for 2 hours at 37°C on a rotating shaker. The cells were passed through a cell strainer (Falcon 352340) and were centrifuged at 400 g for 5 minutes. The cells were suspended in DMEM containing 10% FBS and 2% Penicillin/Streptomycin/ Glutamine, plated at a density of 5 × 10^5 cells/well in a 24-well plate and incubated at 37°C overnight.

Gene-specific RT-PCR analysis

Total RNA was extracted from cultured primary mouse epiphyseal chondrocytes using Rneasy kit (Qiagen 74104). Five micrograms of total RNA were treated with DNA-Free RNA Kit (Zymo Research) and reverse transcribed with Superscript II First Strand Synthesis System for RT-PCR (Invitrogen). All genes were amplified using Taq DNA polymerase (Roche) for 35 cycles and PCR products were fractionated by gel electrophoresis. The primers used for the PCR amplification were 5’CACAGATAAGCCACCAAG3’ and 5’GAC- AACCACCAGGAATGA3’ for Cd31; 5’GGTCTAGGTCGAAGTTTAAAAGTGCCT3’ and 5’TAGGAATGGTATTTGCTGGCGATG- GT3’ for VEGFA1; 5’CTCTGTTGGTGGTGCCTGGCGATTCC- T3’ and 5’GGGATCCACACAGTTGTCTGTT3’ for Vegfr2; 5’GCTGTCGGAGGAGGAAG3’ and 5’TCCCGCTTCTGTCTGTTA3’ for Vegfr3; 5’TCCCGCTTCTGTCTGTTA3’ and 5’GGGATCCACACAGTTGTCTGTT3’ for neuropilin 1; 5’CCCGAACCACCAGAAAG3’ and 5’GAATGCGTCCACACGATTCC- CCA3’ for neuropilin 2.

Results

Generation of mice lacking VEGFA in chondrocytes

In order to study the role of VEGFA in cartilage development we used the loxP-Cre recombination system with the chondrocyte specific Col2al promoter to drive expression of Cre recombinase. Previous attempts to conditionally delete the Vegfa allele in cells expressing collagen type II resulted in lethality at the heterozygous stage around E10 because of defects in development of the dorsal aorta and intersomitic blood vessels, along with defects in heart development (Haigh et al., 2000). We speculated that different Col2al-Cre mouse lines might have different levels and tissue specificities of Cre expression and might therefore allow a conditional deletion of Vegfa in chondrocytes. We crossed floxed-Vegfa mice to animals with Cre expression under the control of the rat Col2al promoter (Schipani et al., 2001). Animals heterozygous for both floxed-Vegfa and Col2al-Cre alleles were collected and mated to floxed-Vegfa homozygous animals. In contrast to what was reported by Haigh et al. (Haigh et al., 2000), we observed no embryonic lethality during these crosses and embryos could therefore be studied at different stages of development.

Lack of VEGFA in chondrocytes leads to impaired embryonic bone development

Staining of skeletons of mice that were heterozygous for both floxed-Vegfa and Col2al-Cre alleles (unaffected) and mice that were heterozygous for Col2al-Cre and homozygous for the floxed-Vegfa alleles (conditional knockout, CKO) with Alizarin
Red and Alcian Blue showed that the Alizarin Red-stained zones were reduced in the VeGfa CKO mice at E18.5, suggesting a reduction in mineralization (Fig. 1). This reduction was observed in long bones in the limbs (Fig. 1B), in ribs (Fig. 1D), in sternum (Fig. 1F), and in the bones of the vertebral column (Fig. 1H). Furthermore, the cartilaginous parts were smaller and stained less intensely for Alcian Blue in the CKO tissues, suggesting a cartilage abnormality.

For more detailed analyses, histological sections were prepared from different skeletal elements at stages E15.5 to E18.5. As can be seen in Fig. 2A,B, in the unaffected mice at E15.5, we observed initiation of blood vessel invasion into the hypertrophic cartilage of long distal limb bones (tibia and fibula), whereas in the VeGfa CKO distal limb bones there was an expansion of the hypertrophic zone, so that it occupied most of the diaphysis, and no evidence of blood vessel invasion and trabecular bone formation could be seen. In order to document the differences in the invasion of blood vessels into the hypertrophic zone between unaffected and VeGfa CKO mice we stained for PECAM (CD31), an endothelial cell marker. At E16.0 (Fig. 2C,D), in unaffected tibia we observed abundant CD31 staining throughout the periosteum and in the marrow cavity. In the VeGfa CKO mice (D), there are vessels in the periosteum but no apparent invasion into the hypertrophic zone. In the radius and ulna at E16.5 in unaffected (E) mice, bone marrow and bone trabeculae are present below the hypertrophic zone (arrowhead). In VeGfa CKO mice the growth plate contains a much longer hypertrophic zone (F, arrowhead). At E18.5, unaffected ribs (G) contain a shorter hypertrophic zone. In contrast, the VeGfa CKO ribs contain a greatly expanded zone of hypertrophy (H, arrowhead).
adjacent to the growth plate. In contrast, in tibia of Vegf a CKO mice there was clearly no vessel invasion into cartilage. At E16.5 (Fig. 2E,F), a marrow cavity was established in the distal limb bones of unaffected and Vegf a CKO mice, but despite this evidence of a normal architecture, the zones of hypertrophic chondrocytes remained larger in Vegf a CKO than in unaffected growth plates. Not only limb bones were affected in the Vegf a CKO mice; expansion of the hypertrophic zone could also be observed at E18.5 in ribs (Fig. 2G,H). These results suggest that VEGFA is important both for the initiation of blood vessel invasion into hypertrophic cartilage as well as later in the process of endochondral bone formation.

Lack of VEGFA in chondrocytes leads to reduction in the removal of terminally differentiated hypertrophic chondrocytes

Expansion of the hypertrophic zone in the Vegf a null growth plates (Fig. 3A,B) could be a consequence of either accelerated chondrocyte differentiation or delayed removal of terminally differentiated hypertrophic chondrocytes. To distinguish between these possibilities, we studied chondrocyte differentiation by examining the expression of several chondrocyte differentiation markers in the humerus of E16.5 mice.

The expression of collagen II, a marker for resting and proliferating chondrocytes, did not show any difference between Vegf a CKO and unaffected mice (Fig. 3C,D). Expression of collagen X, a marker for hypertrophic chondrocytes, was similar in the unaffected and Vegf a CKO bones (Fig. 3E,F), although, as mentioned above, the hypertrophic zones in the mutant bones were larger than those of wild-type bones. This suggests that some of the hypertrophic chondrocytes in the mutant bones are differentiated to terminal hypertrophic chondrocytes (with a low level of collagen X expression) at E16.5. We also studied the expression of osteopontin (Osp), a marker for terminally differentiated chondrocytes. As can be seen in Fig. 3G,H, Vegf a CKO bones contained several rows of chondrocytes expressing Osp, whereas expression was restricted to only one row of chondrocytes in the unaffected bones. These results suggest that there is delayed removal of terminally differentiated hypertrophic chondrocytes in the Vegf a CKO growth plates. This is consistent with the overall decrease in cartilage angiogenesis in the Vegf a CKO mice (see above).

Loss of VEGFA in chondrocytes results in massive cell death

Histological studies of Vegf a CKO femurs at E16.5 revealed a zone of large, balloon-like cells, poorly stained and with a ‘ghost’-like appearance, at the center of the epihyysis (Fig. 4A,B). There was some variation in the severity of the abnormalities, with the distal limb bones showing the highest degree of abnormality. In the distal bones at E18.5, extensive regions of dead cells were observed (Fig. 4C,D). These regions of cell death were located in the central regions of the skeletal elements, starting at an articular surface and continuing through the resting to the proliferating zones of chondrocytes and ending in a misshapen growth plate. Depending on the severity of the phenotype, the growth plate was dramatically affected and hypertrophic chondrocytes of normal shape were almost completely missing. Outside the center of the growth plates, proliferating cells appeared to accumulate (Fig. 4D). These zones of proliferating cells were much more extended in Vegf a CKO than in unaffected growth plates.
In order to study the nature of the cell death, we assayed unaffected and Vegfa CKO bones for TUNEL-positive cells. As can be seen in Fig. 5, sections of Vegfa CKO growth plates revealed cells with shrunken cytoplasm and condensed nuclei, similar to that observed for apoptotic cells (Fig. 5A-D). Strong TUNEL-positive signals were seen in regions in which we observed dead cells histologically. Interestingly, many of the TUNEL-positive cells flanked the zones of dead cells (Fig. 5F,G). We failed to observe TUNEL-positive cells in growth plates of unaffected mice (Fig. 5E). Previous attempts to conditionally delete Vegfa in cells expressing collagen type II resulted in lethality and defects in heart development at stage E10 (Haigh et al., 2000). Malfunction of the heart can reduce the supply of nutrients and oxygen to the developing embryo, leading to indirect effects on skeletal development. To exclude such an indirect effect, we crossed the floxed-Vegfa mice to animals with Cre expression under the control of the rat Prx1 promoter. This line of Cre-mice expresses the Cre recombinase in limb bud mesenchyme (Logan et al., 2002). Heterozygous animals for both floxed-Vegfa and Prx1-Cre alleles were collected and mated to floxed-Vegfa homozygous animals. Animals homozygous for floxed-Vegfa and heterozygous for Prx1-Cre (Prx1/Vegfa CKO; a detailed description of these mice will be presented elsewhere) had dramatically smaller and misshapen bones. At E16.5 the epiphyses of limb bones of Prx1/Vegfa CKO mice contained extensive regions of dead cells. In some cases (Fig. 5H), the head of the humerus was composed entirely of dead cells. These results suggest that VEGFA is necessary for chondrocyte survival during endochondral bone formation.

**Hif1a differentially regulates Vegfa expression during bone development**

The extensive cell death in the epiphyses of Col2a1/Vegfa CKO and Prx1/Vegfa CKO null bones had a striking similarity to the phenotype previously observed when Hif1a expression was abolished in developing cartilage (Schipani et al., 2001). In the case of Hif1a CKO mice, it was also demonstrated that extensive apoptosis occurs in cartilage epiphyses. To study the similarities between the two phenotypes in more detail, we crossed floxed-Hif1a mice to Col2a1-Cre mice. Animals heterozygous for both floxed-Hif1a and Col2a1-Cre alleles were collected and mated to floxed-Hif1a homozygous animals. Histological sections of Hif1a CKO hind limbs at E15.5 revealed robust signs of cell death in the center of long bone epiphyses (Fig. 4E,F). Furthermore, we observed a delay in vessel invasion into primary ossification centers (Fig. 4A,B). Since Hif1a is a known regulator of Vegfa expression, one possibility to explain this delay in vessel invasion would be a reduction of Vegfa expression in Hif1a CKO primary ossification centers. To study the possibility that Hif1a regulates the expression of Vegfa in the developing bone, we examined Vegfa expression in unaffected and Hif1a CKO bones. During cartilage development, Vegfa shows a dynamic pattern of expression. During the establishment of primary ossification centers, Vegfa is expressed primarily in the hypertrophic zone (Fig. 6C). Later, as development proceeds, Vegfa expression in the hypertrophic zone is maintained, and a moderate level of Vegfa expression can be detected in the epiphyses (Fig. 6E). As can be seen in Fig. 6D, at E15.5 Vegfa expression is detectable in the hypertrophic zone of tibia of Hif1a CKO mice. In contrast, Vegfa expression in the Hif1a CKO epiphysis is dramatically reduced at E18.5 (Fig. 6F). This result suggests that at E15.5 the expression of Vegfa in hypertrophic chondrocytes is not Hif1a-dependent, while Vegfa expression in the epiphyses is Hif1a-dependent later in development. In order to identify a receptor that might mediate VEGFA signaling in epiphyseal chondrocytes, we examined the expression of the known receptors of VEGFA in these cells. We failed to detect expression of Vegfr1 and Vegfr2, but were able to identify expression of Vegfr3, neuropilin 1 (Nrp1) and neuropilin 2 (Nrp2) (Fig. 7), suggesting that these receptors...
might be involved in mediating the effects of VEGFA in epiphyseal chondrocytes.

**HIF1α is involved in chondrocyte differentiation**

Studying sections of E15.5 Hif1a CKO tissues revealed, as was mentioned above, a delay in vessel invasion into primary ossification centers (Fig. 6A,B). The detectable Vegf expression in Hif1a CKO hypertrophic chondrocytes (Fig. 6D) suggests that delay in vessel invasion into the Hif1a CKO hypertrophic cartilage template is not a simple consequence of a reduction in Vegf expression. HIF1α may therefore play a role in chondrocyte differentiation. To analyze this possibility further,
we studied chondrocyte differentiation in Hif1a CKO mice by following the temporal expression of cartilage differentiation markers (Fig. 8). At E15.5, Col10a1 expression was reduced in the most mature (terminally differentiated) hypertrophic chondrocytes of unaffected bones. In contrast, in the Hif1a CKO tissue the expression of Col10a1 was still prominent in the hypertrophic zone (Fig. 8A,B). Differences in the expression of Pthr1 and Ihh further support the possibility that differentiation is delayed in Hif1a CKO chondrocytes. In the unaffected bones, the expression of these two markers was reduced in the terminally differentiated hypertrophic chondrocytes, but most of the hypertrophic chondrocytes still expressed Pthr1 and Ihh in the Hif1a CKO bones (Fig. 8C-F). These results indicate that HIF1α has a role in cartilage differentiation within long bone growth plates.

**Normal vascularization outside Vegfa null cartilaginous templates**

The extensive cell death in the Vegfa null cartilaginous templates could be the result of a reduced vascularization in the vicinity of developing bones. In order to study this possibility we performed immunohistochemical studies of blood vessels surrounding the knee joint. This region was selected because it showed a strong cell death phenotype (Fig. 4D). We chose to study sections of E16.0 embryos since we wanted to insure that any difference in vascularization would precede the cell death phenotype. Serial sections of unaffected and Vegfa CKO knee joints at stage E16.0 were stained with CD31 antibodies for visualization of blood vessels (Fig. 9). Analysis of comparable sections did not reveal any apparent differences between unaffected and Vegfa CKO sections (Fig. 9A,B). Moreover, we were able to identify regions with extensive vascularization in the vicinity of both diaphyses and epiphyses. In some cases we were able to observe blood vessels in rather close proximity to areas showing the typical appearance of cell death (Fig. 9B,D). These results suggest that in Vegfa CKO bones there is no apparent reduction in the vascularization of tissues surrounding the cell death regions of cartilaginous templates.

**Abnormal pattern of expression of Ihh and Pthr1 in Vegfa null growth plates**

Further similarities between the Hif1a and Vegfa conditional null phenotypes could be observed by histological examination of growth plates at E18.5. In both cases, the proliferating zones were divided by a zone of dead cells. In unaffected growth plates, the proliferating zone was clearly defined and extended to the center of the epiphysis. In contrast, in the Hif1a and Vegfa CKO growth plates, the proliferating cells extended further into the epiphysis (Fig. 10A,B) (Schipani et al., 2001). In order to get insights into a possible mechanism for this abnormality, we examined the Vegfa CKO growth plates for expression of Ihh and Pthr1, two genes that are known to have roles in the regulation of chondrocyte proliferation and differentiation to hypertrophy.

As can be seen in Fig. 10C,D, the expression of Col2a1 at E18.5 in the humerus of Vegfa CKO mice defined the cell death region as the zone in which Col2a1 expression was lost. These dying or dead cells also lost the expression of Pthr1 and Ihh. Flanking the regions of dead cells, we identified expanded regions of proliferating cells that also expressed Ihh and Pthr1 (Fig. 10E-H). It is possible that this change in the expression pattern of Ihh and Pthr1 may lead to the formation of an extended zone of proliferating chondrocytes that flanks the regions of dead cells in the Vegfa CKO growth plates.

**Discussion**

In this paper we provide, for the first time, evidence that VEGFA is necessary for chondrocyte survival in addition to its known role in regulating angiogenesis during endochondral
bone formation. Comparing the skeletal phenotypes of mice with chondrocyte specific deletion of either Vegfa or Hif1a reveals a large similarity between the phenotypes. This suggests that HIF1α, a well-known regulator of VEGFA, and VEGFA are critical for chondrocyte survival during bone formation.

**Hif1α and Vegfa are part of a chondrocyte survival pathway**

HIF1α regulates the transcription of a broad range of genes that are involved in a variety of processes such as glucose metabolism, angiogenesis and cell survival (Pugh and Ratcliffe, 2003; Semenza, 2003). Several stimuli, such as hypoxia, hormones and growth factors, induce stabilization of the HIF1 heterodimeric transcription complex. This complex is composed of HIF1α and HIF1β. HIF1β is constitutively expressed, whereas HIF1α is tightly regulated (Maxwell et al., 1993; Semenza and Wang, 1992; Wang and Semenza, 1993; Wang and Semenza, 1995; Zelzer et al., 1998); the tumor-suppressor protein von Hippel-Lindau (VHL) protein is a key element in this regulation (Bruick and McKnight, 2001; Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001; Yu et al., 2001).

HIF1α is known to be a regulator of VEGFA in many systems (Shima et al., 1995). However, in hypertrophic chondrocytes of Hif1a CKO growth plates, Vegfa expression was comparable to the expression in unaffected, control growth plates at E15.5 (Fig. 6). This suggests that Vegfa expression is not dependent on Hif1a in hypertrophic chondrocytes, at least at that stage of development. Runx2 and Ctgf are two genes that may be involved in the regulation of VEGFA at that stage or later, since Vegfa expression in the hypertrophic zones of Runx2 and Ctgf null embryos is decreased (Ivkovic et al., 2003; Zelzer et al., 2001) (Fig. 11). In epiphyseal chondrocytes Hif1a may well be a major regulator of Vegfa expression, since the expression of Vegfa was dramatically reduced at E18.5 in Hif1a CKO bones (Fig. 6F). The important role of Hif1a in regulating expression of Vegfa in epiphyseal chondrocytes is further demonstrated by the recent analysis of the phenotype of mice that are homozygous for VHL null alleles in chondrocytes (Pfander et al., 2004). In these VHL conditional knockout mice, levels of hydroxylated HIF1α protein are elevated and expression of Vegfa is enhanced in epiphyseal cartilage.

The striking homology between the Hif1a and Vegfa CKO cartilage phenotypes (Fig. 4) and the loss of VEGFA production under hypoxic conditions in cultured Hif1a-null epiphyseal chondrocytes (Pfander et al., 2003) further support the hypothesis that HIF1α regulates Vegfa expression in a chondrocyte survival pathway (Fig. 11). Interestingly, there is a time difference in the appearance of the cell death phenotype in the two conditional knockouts. The cell death phenotype appears at E14.5 in bones of Hif1a CKO mice (Schipani et al., 2001) but becomes apparent two days later in Vegfa CKO animals (Fig. 4). There can be several explanations for this difference. First, HIF1α is known to regulate the expression of many genes that have a role in cell survival, including Igf2 and Tgfα (Feldser et al., 1999; Krishnamachary et al., 2003) and Vegfa could be one of these genes required for cell survival at E14.5, even at low, almost undetectable, levels of expression. A second possibility is that the cell survival function of HIF1α is independent of VEGFA at E14.5 but becomes dependent on VEGFA later at E16.5, when Vegfa expression in epiphyseal chondrocytes can be detected (Fig. 6).

Although VEGFA is best known for its activity as an angiogenic factor (Ferrara et al., 2003), it has been shown to be a survival factor for endothelial and hematopoietic stem cells (Benjamin et al., 1999; Gerber et al., 1998a; Gerber et al., 2002; Gerber et al., 1998b), and here we describe a role for VEGFA in supporting chondrocyte survival. We cannot at present rule out the possibility that Vegfa expression in
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The epiphyseal chondrocytes regulates some aspect of vascularization in the vicinity of the epiphysis (with secondary consequences for the survival of epiphyseal chondrocytes), but we favor the view that VEGFA has a direct role in chondrocyte survival. First, epiphyseal chondrocytes express three receptors for VEGFA, namely Vegfr3, Nrp1 and Nrp2, suggesting that VEGFA can initiate signaling in these cells. Interestingly, it was recently demonstrated that Nrp1 can, independently of other receptors, initiate cell signaling (Bachelder et al., 2001; Foster et al., 2003; Wang et al., 2003). Secondly, cell death in the Prx1/Vegfa CKO epiphyseal cartilage was observed at E16.5 (Fig. 5H), the stage at which we identified cell death in the Col2a1/Vegfa CKO mice, suggesting that VEGFA is needed for chondrocyte survival at a specific developmental time. This finding is particularly important, since the cartilaginous elements in the Prx1/Vegfa CKO are significantly smaller than in the Col2a1/Vegfa CKO mice, suggesting that the cell death cannot be a simple consequence of a deficient diffusion of nutrients and oxygen into the epiphysis. Consistent with this interpretation is the recent report that incubation of chondrocytes in hypoxic conditions did not cause cell death (Pfander et al., 2003). Finally, when we analyzed vascularization in the vicinity of the cartilaginous elements to examine a possible reduction in vessel numbers before cell death occurs in the Vegfa CKO mice, we failed to observe any obvious difference between unaffected and Vegfa CKO limbs.

Several studies have recently suggested a role for VEGFA during organ development as a mediator of cell-cell interactions between endothelial and parenchymal cells (Cleaver and Melton, 2003). We have therefore considered the possibility that the role of VEGFA in supporting survival of
epiphyseal chondrocytes may somehow involve an interaction with endothelial cells in the vicinity of the cartilage. During development of pancreas and liver there is a close physical interaction between endothelial and endodermal cells and VEGFA was demonstrated to have an important role in interactions that led to development of these organs (Lammert et al., 2001; Matsumoto et al., 2001). Since chondrocyte differentiation and cartilage formation take place in an endothelium-free environment, any involvement of endothelial cells in the HIF1α/VEGFA chondrocyte survival pathway would have to include long-range interactions.

**VEGFA regulation of bone vascularity**

Blood vessel invasion into the primary ossification center is a key step in bone development. In this study we have provided further in-vivo evidence that VEGFA is required for angiogenesis into the primary ossification center and the maintenance of blood vessel growth in developing bones. At E15, vessels are invading hypertrophic cartilage, and as development proceeds, the vessels continue to grow under the growth plates as hypertrophic chondrocytes are being removed. In the Vegfa CKO bones, there is a delay in blood vessel invasion into the primary ossification center (Fig. 2), and accumulation of terminally differentiated chondrocytes in growth plates suggests a reduction of vessel sprouting and cartilage removal (Fig. 3). Since the angiogenic process in the Vegfa CKO bones was not completely abolished, we consider the possibility that VEGFA is not the only factor regulating the process of endochondral angiogenesis, although it is also possible that the Cre was less than fully efficient in generating a chondrocyte-specific Vegfα null phenotype. However, comparing the phenotypes of the Vegfa CKO and the Vegfa120 mice clearly demonstrates that the 120 isoform cannot compensate for the loss of the other VEGFA isoforms as an angiogenic regulator in endochondral bones, since the conditional loss of Vegfα in chondrocytes results in an angiogenic phenotype similar to the phenotype observed in mice that express only the 120 isoform (Fig. 2) (Maes et al., 2002; Zelzer et al., 2002). In contrast, the 120 isoform is clearly sufficient to compensate for the loss of other isoforms as a survival factor for chondrocytes, since the massive cell death observed in Vegfa CKO bones was not observed in Vegfa120 mice (Maes et al., 2002; Zelzer et al., 2002). Runx2 null bones represent the most severe example of a deficient angiogenic process in developing bones, since the hypertrophic zone is not invaded by blood vessels (Zelzer et al., 2001). This lack of endochondral angiogenesis in Runx2 null mice makes the mice useful for further studies aimed at identifying other components of the mechanism that regulates skeletal vascularization.

In conclusion, in this study we provide further in-vivo evidence for the important role of VEGFA in blood vessel invasion into hypertrophic cartilage during bone development. More importantly, we describe for the first time a connection between VEGFA and chondrocyte survival during skeletal development. The similarities in the phenotypes of Hif1α and Vegfα null bones, together with the in-vivo finding that HIF1α is a regulator of Vegfα expression, establishes that these two genes are part of a pathway that regulates chondrocyte survival.

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