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 (*Hif1a*) expression was abolished in developing cartilage. This similarity of cell death phenotypes and the deficient VEGFA production in *Hif1a* 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 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 *Vegfa* or hypoxia-inducible factor 1α (*Hif1a*) 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-Hif1a* (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 CO_2. 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 procedures. 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 ^{33}P-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′CACAGATAAGCCCACCAGAG3′ and 5′GACAACCACCGCAATGA3′ for *Cd31*; 5′GGCTCAGGGTCGAAGTTAAAAGTGCCT3′ and 5′TAGGATTGTATTGGTCTGCCGATGGGT3′ for VEGFAr1; 5′CTCTGTGGGTTTGCCTGGCGATTTTCT3′ and 5′GCGGATCACCACAGTTTTGTTCTTGTT3′ for *Vegfr2*; 5′GCTGGCAGGAGGAGGAA3′ and 5′TCCCGCTGTCTGTCTGGTTA3′ for *Vegfr3*; 5′TCCCGCCTGAACTACCCTGAA3′ and 5′GCCTTGCGCTTGCTGTCATC3′ for neuropilin 1; 5′CCCCGAACCCAACCAGAAGA3′ and 5′GAATGCCATCCCAGATGTCCA3′ 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 *Col2a1* 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 *Col2a1-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 *Col2a1* promoter (Schipani et al., 2001). Animals heterozygous for both *floxed-Vegfa* and *Col2a1-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 *Col2a1-Cre* alleles (unaffected) and mice that were heterozygous for *Col2a1-Cre* and homozygous for the *floxed-Vegfa* alleles (conditional knockout, CKO) with Alizarin

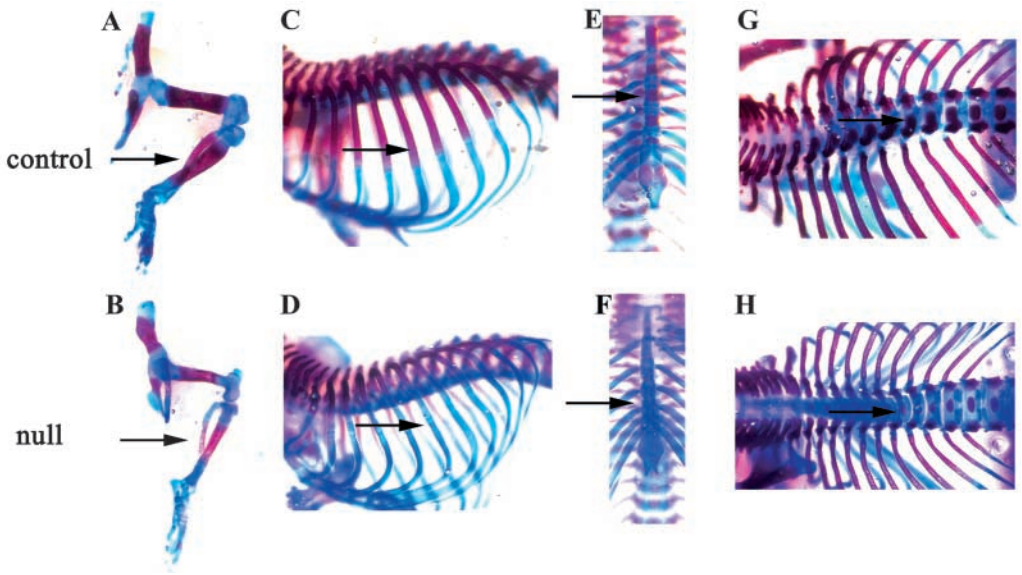

Fig. 1. Reduced skeletal mineralization in *Vegfa* conditional knockout (CKO) mice. A comparison of the skeletons of unaffected (A,C,E,G) and *Vegfa* CKO (B,D,F,H) mice reveals reduced size of areas stained with Alizarin Red, suggesting reduced mineralization of mutant bones. Regions significantly affected (arrows) include the bones in hind limbs (A,B), ribs (C,D), sternum (E,F), and vertebral column (G,H).

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 diaphysis and capillary network

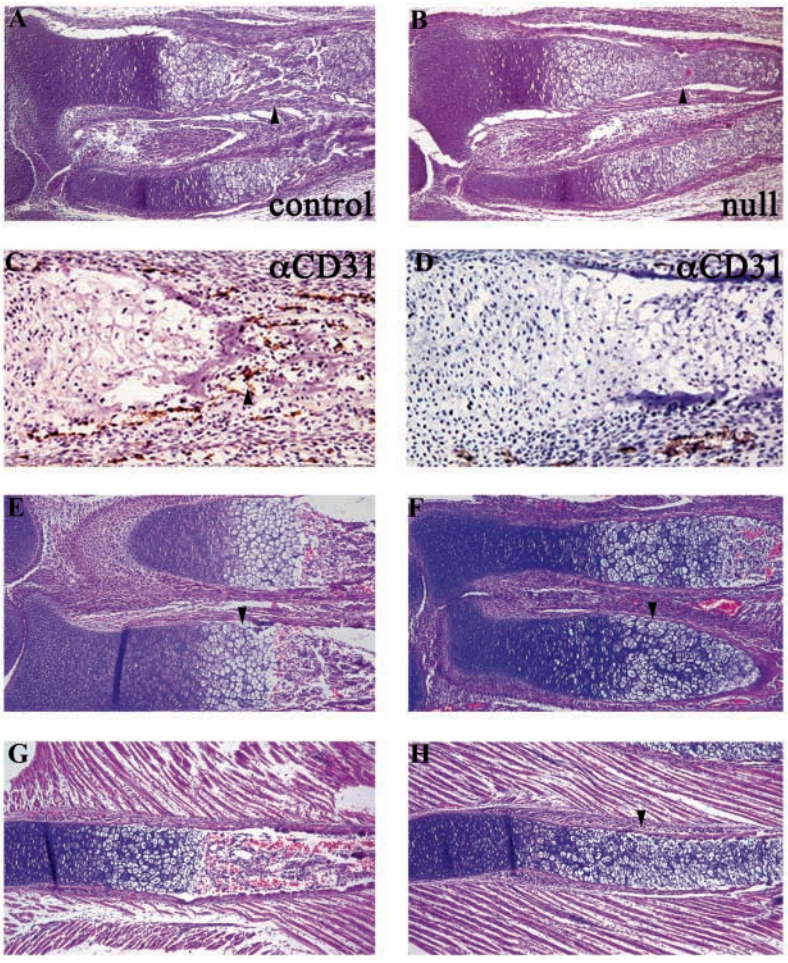

Fig. 2. Reduced angiogenesis in *Vegfa* conditional knockout (CKO) bones. Histology of unaffected and *Vegfa* CKO mice identifies marked differences in skeletal elements during development. In tibia and fibula at E15.5 in unaffected mice (A), blood vessel invasion into the hypertrophic cartilage and marrow cavity can be observed (arrowheads), while no invasion can be seen into hypertrophic cartilage in the *Vegfa* CKO mice (B). CD31 immunostaining of tibia at E16.0 in unaffected mice (C) shows vessels 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).

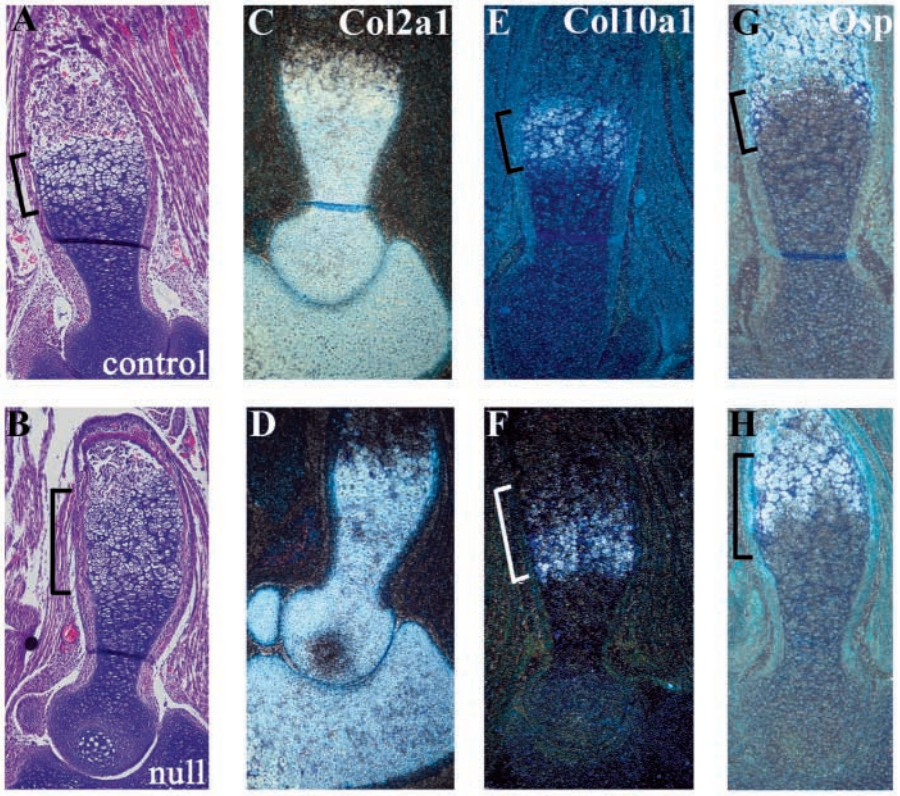

Fig. 3. Reduced removal of terminally differentiated chondrocytes in *Vegfa* conditional knockout (CKO) bones. Histological sections of E16.5 unaffected (A) and *Vegfa* CKO (B) humerus show the presence of an expanded hypertrophic zone in the *Vegfa* CKO growth plate (defined by brackets). *Col2a1* expression is seen throughout the cartilage anlagen in both unaffected (C) and *Vegfa* CKO (D) mice. In unaffected (E) mice *Col10a1* expression is seen in the hypertrophic zone (bracket) while in *Vegfa* CKO (F) mice, *Col10a1* expression is seen only in part of the hypertrophic zone (bracket), suggesting that some of the hypertrophic chondrocytes are differentiated into terminal hypertrophic chondrocytes. *Osp* expression is seen in the last row of terminally differentiated hypertrophic chondrocytes and extensively in the osteoblasts under the growth plate in the unaffected mice (G), while in the *Vegfa* CKO growth plate, *Osp* expression is detected in several rows of terminally differentiated hypertrophic chondrocytes (H).

adjacent to the growth plate. In contrast, in tibia of *Vegfa* 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 *Vegfa* CKO mice, but despite this evidence of a normal architecture, the zones of hypertrophic chondrocytes remained larger in *Vegfa* CKO than in unaffected growth plates. Not only limb bones were affected in the *Vegfa* 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 *Vegfa* 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 *Vegfa* CKO and unaffected mice (Fig. 3C,D). Expression of collagen X, a marker for hypertrophic chondrocytes, was similar in the unaffected and *Vegfa* 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, *Vegfa* 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 *Vegfa* CKO growth plates. This is consistent with the overall decrease in cartilage angiogenesis in the *Vegfa* CKO mice (see above).

Loss of VEGFA in chondrocytes results in massive cell death

Histological studies of *Vegfa* 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 epiphysis (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 *Vegfa* CKO than in unaffected growth plates.

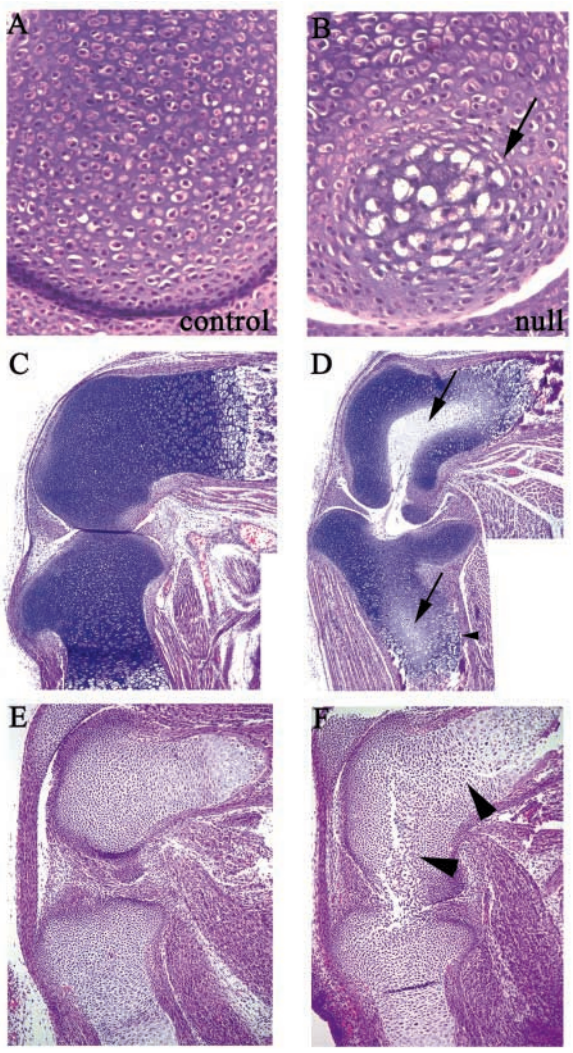

Fig. 4. Cell death in *Vegfa* and *Hif1a* conditional knockout (CKO) bones. Histological sections of E16.5 *Vegfa* CKO humerus (B) show areas of cell death (arrow), unlike the unaffected bone (A). Compared with the femur and tibia of unaffected mice (C), the E18.5 *Vegfa* CKO bones (D) were misshapen with extensive regions of dead cells in the center of the bones, starting at the articular surface and continuing through the resting to the proliferating zones of chondrocytes and ending in a misshapen growth plate (arrows). Histological sections of unaffected (E) and *Hif1a* CKO (F) femur and tibia at E15.5 show misshapen bones with extensive regions of dead cells in the center of the bones (arrowheads).

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. 6A,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

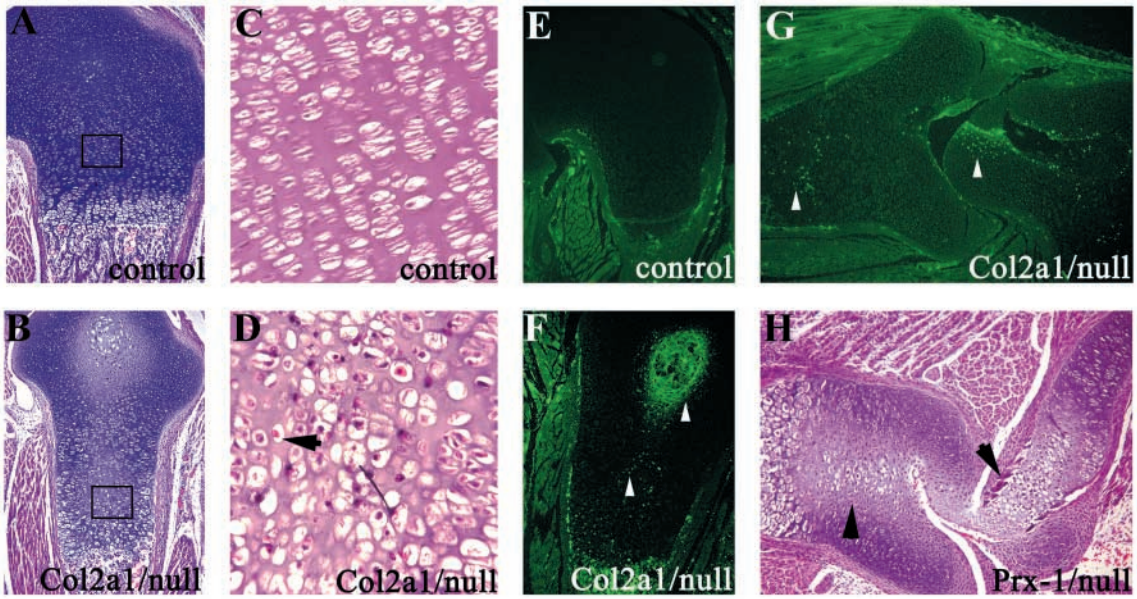

Fig. 5. Apoptosis in *Vegfa* conditional knockout (CKO) bones. Histological sections through the center of E18.5 unaffected (A,C) and *Vegfa* CKO (B,D) humerus. Areas within the squares in A and B are shown at high magnification in C and D. In the affected epiphysis (D), the presence of apoptotic cells with shrunken cytoplasm and condensed nuclei is observed. TUNEL assay of E18.5 unaffected (E) and *Vegfa* CKO (F) humerus and *Vegfa* CKO tibia and femur (G) shows strong TUNEL-positive signals in regions of apoptotic cells (arrowheads) in the *Vegfa* CKO bones (E and F are serial sections of A and B, while G is serial section of Fig. 4 D). Histological sections of E16.5 *Prx1*/*Vegfa* CKO scapula and humerus show misshapen bones with extensive regions of dead cells (H, arrowheads).

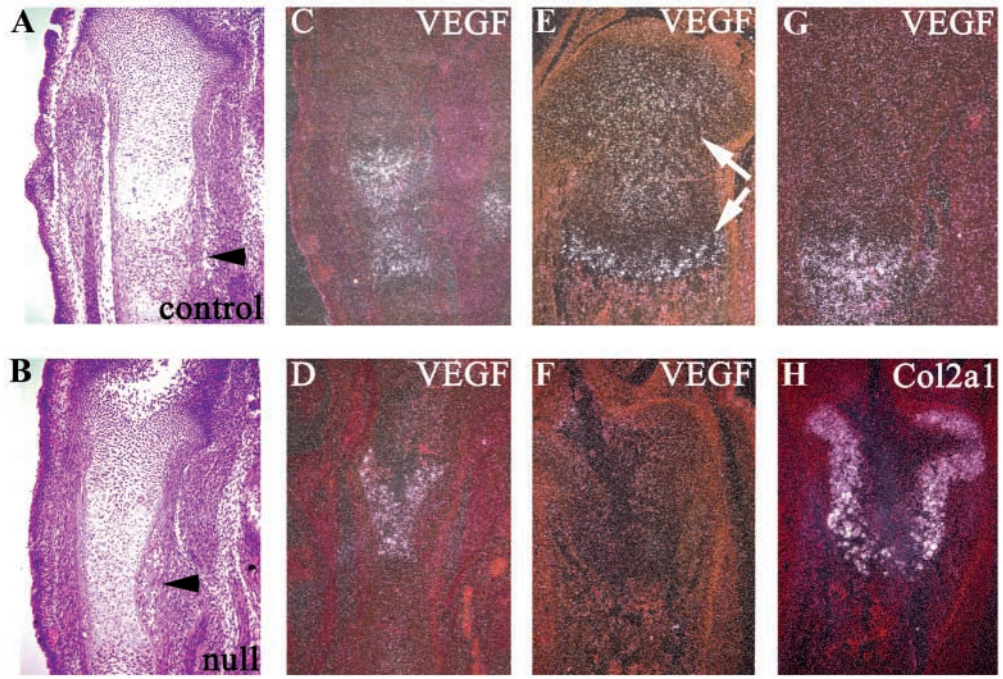

Fig. 6. Analysis of *Vegfa* expression in *Hif1a* conditional knockout (CKO) mice. Histological sections of E15.5 tibia of unaffected (A) and *Hif1a* CKO mice (B) reveal a delay in vessel invasion into the *Hif1a* CKO primary ossification center (arrowheads). *Vegfa* expression in the primary ossification center of unaffected tibia at E15.5 (G) shows expression in hypertrophic chondrocytes but no detectable expression in the epiphysis. At E18.5 (E), the expression of *Vegfa* in the hypertrophic zone is maintained and it is possible to detect a moderate level of *Vegfa* expression in the epiphysis (arrows). At E15.5 in the primary ossification center of tibia there is no apparent difference in *Vegfa* expression in the hypertrophic chondrocytes of unaffected (C) and *Hif1a* CKO mice (D), although the shape of the hypertrophic region is abnormal in the *Hif1a* CKO section (D). At E18.5 (F) the expression of *Vegfa* in the *Hif1a* CKO is dramatically decreased. At this stage, the extensive cell death in the *Hif1a* tissue makes it almost impossible to see any hypertrophic zone of chondrocytes and all remaining viable cells are proliferating *Col2a1*-positive cells (H).

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 *Vegfa* 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 *Vegfa* expression. HIF1α may therefore play a role in chondrocyte differentiation. To analyze this possibility further,

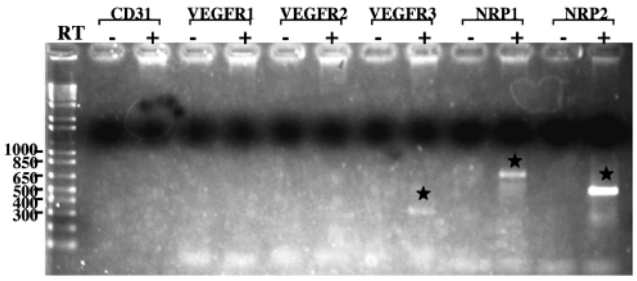

Fig. 7. Gene specific RT-PCR analyses of VEGFA receptors in epiphyseal chondrocytes. Total RNA was extracted from epiphyseal chondrocytes. RT-PCR reactions were performed in the presence of reverse transcriptase (RT) (+) or in its absence (–) (as a control for genomic DNA contamination). *Cd31* was also amplified as a control for endothelial cell contamination. Nucleotide size markers are indicated at left. Amplified bands are only seen in the *Vegfr3*, *Nrp1* and *Nrp2* lanes, as indicated by asterisks.

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 chondrocyte differentiation within long bone growth plates.

Normal vascularization outside *Vegfa* null cartilaginous templates

The extensive cell death in the *Vegfa* CKO epiphyses 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

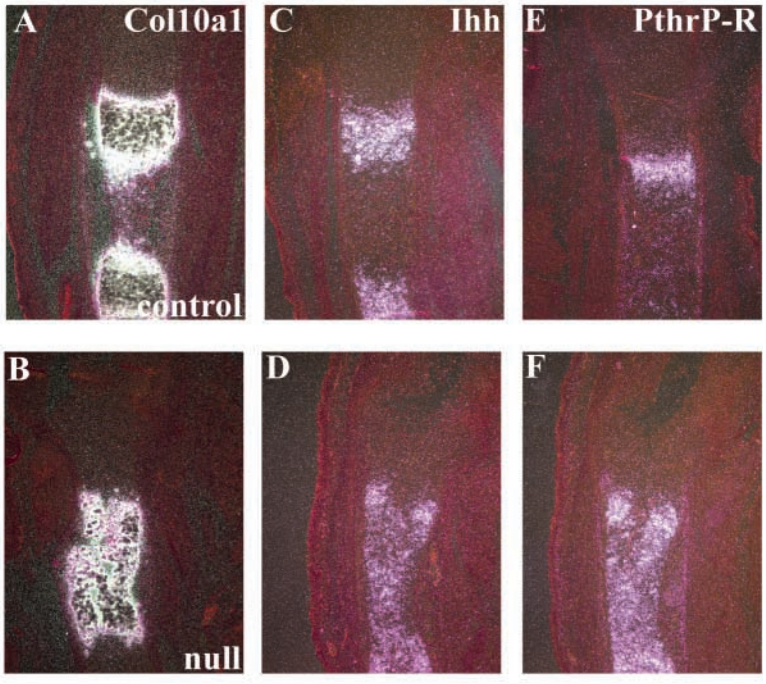

Fig. 8. Analysis of chondrocyte differentiation in *Hif1a* conditional knockout (CKO) mice. *Col10a1*, *Ihh* and *Pthr1* expression domains are divided by cells that have lost the expression of these markers in the growth plates of unaffected (A,C,E) mice, while the *Hif1a* CKO (B,D,F) mice cells in the center of the hypertrophic domains express all three markers.

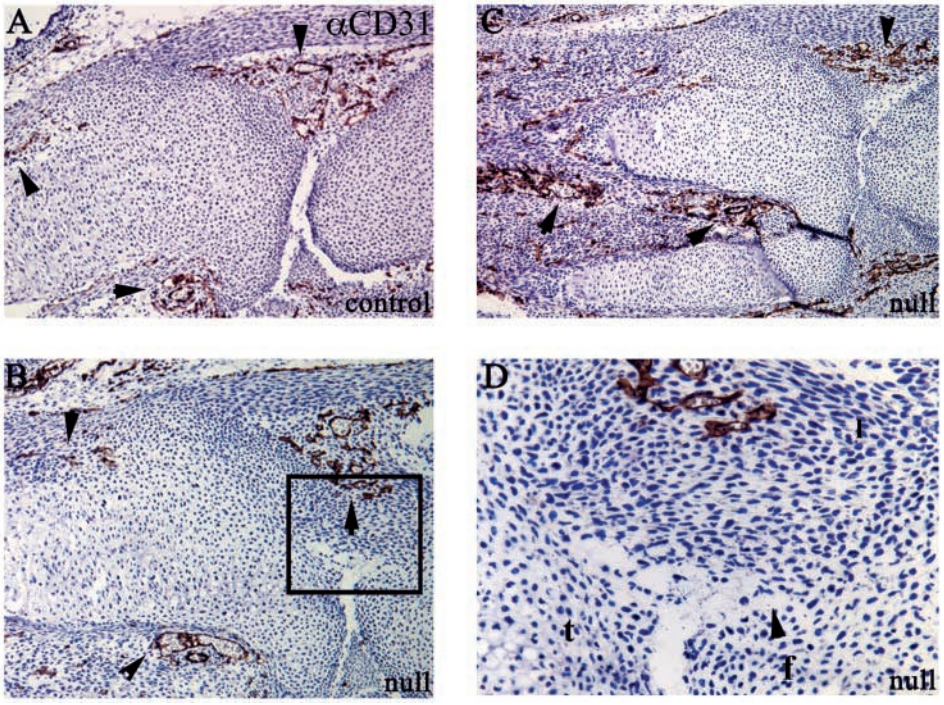

Fig. 9. Vascularization in the vicinity of *Vegfa* conditional knockout (CKO) cartilaginous elements. CD31 immunostaining of unaffected (A) and *Vegfa* CKO (B,C,D) tibias at E16.0. Comparison of histologically comparable sections of unaffected (A) and *Vegfa* CKO (B) knee joint regions reveals no major difference in CD31 immunostaining (arrowheads). Extensive vascularization in the vicinity of the diaphyses and the epiphyses of *Vegfa* CKO knee joint region (C, arowheads). In D, an area indicated by a square in B shown at high magnification, CD31-positive blood vessels are seen in the vicinity of a region of initial cell death between the femur (f), tibia (t), and the anterior cruciate ligament (l).

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 *Tgfa* (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

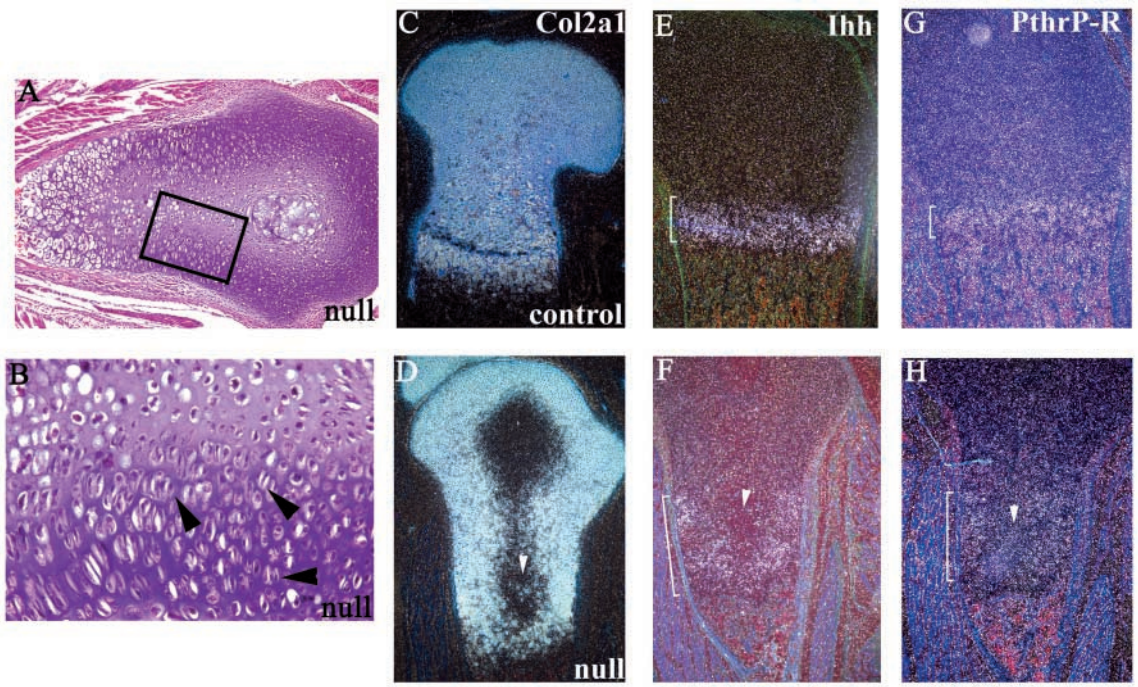

Fig. 10. Expression of *Col2a1*, *Ihh*, and *Pthr1*, in the E18.5 humerus. In *Vegfa* conditional knockout (CKO) mice (A), areas of increased proliferation (square) can be seen surrounding areas of cell death; square in A seen at higher magnification in B, arrowheads indicate proliferating cells. *Col2a1* expression is seen throughout the cartilage in unaffected mice (C) while in *Vegfa* CKO (D) mice *Col2a1* expression is lacking in the center of the cartilage (arrowhead). *Ihh* and *Pthr1* expression in the unaffected humerus (E,G) is restricted to a narrow strip of prehypertrophic cells. In the *Vegfa* CKO growth plate the domain of expression of both *Ihh* and *Pthr1* is expanded (F,H).

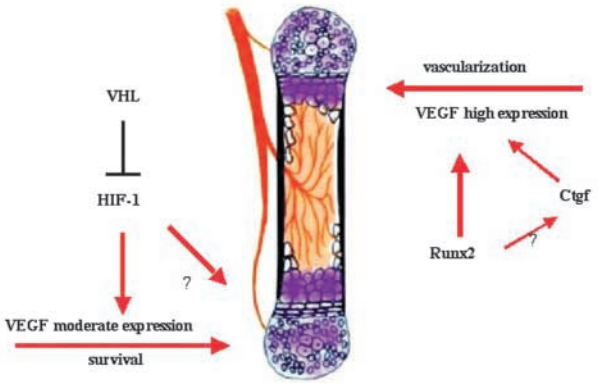

Fig. 11. Two distinct roles of VEGFA during endochondral bone formation are regulated by different pathways. Diagram showing major components of the pathways that regulate expression of *Vegfa* in hypertrophic chondrocytes (right) and epiphyseal chondrocytes (left) during endochondral ossification. Angiogenesis into hypertrophic cartilage and establishment of the primary ossification center in developing long bones depend on a high level of *Vegfa* expression, controlled by the transcription factor Runx2, in hypertrophic chondrocytes (Zelzer et al., 2001). Since a reduction in the level of *Vegfa* was also observed in *Ctgf* null mice (Ivkovic et al., 2003), it is possible that Runx2 and Ctgf may be components of two independent or dependent regulatory pathways for control of *Vegfa* expression. In epiphyseal cartilage, cell survival depends on expression of *Vegfa* at a moderate level under the control of HIF1 and von Hippel-Lindau (VHL) protein. Whether *Vegfa* is the only critical target gene for HIF1 regulation in this chondrocyte survival pathway is not known, and it is possible that other genes may also play a role.

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 *Vegfa* 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 *Vegfa* 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 *Hif1a* and *Vegfa* null bones, together with the in-vivo finding that HIF1α is a regulator of *Vegfa* expression, establishes that these two genes are part of a pathway that regulates chondrocyte survival.

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