Development 129, 1893-1904 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV2824

Skeletal defects in VEGF$^{120/120}$ mice reveal multiple roles for VEGF in skeletogenesis

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Accepted 22 January 2002

SUMMARY

Angiogenesis is an essential component of skeletal development and VEGF signaling plays an important if not pivotal role in this process. Previous attempts to examine the roles of VEGF in vivo have been largely unsuccessful because deletion of even one VEGF allele leads to embryonic lethality before skeletal development is initiated. The availability of mice expressing only the VEGF120 isoform (which do survive to term) has offered an opportunity to explore the function of VEGF during embryonic skeletal development. Our study of these mice provides new in vivo evidence for multiple important roles of VEGF in both endochondral and intramembranous bone formation, as well as some insights into isoform-specific functions. There are two key differences in vascularization of developing bones between wild-type and VEGF$^{120/120}$ mice. VEGF$^{120/120}$ mice have not only a delayed recruitment of blood vessels into the perichondrium but also show delayed invasion of vessels into the primary ossification center, demonstrating a significant role of VEGF at both an early and late stage of cartilage vascularization. These findings are the basis for a two-step model of VEGF-controlled vascularization of the developing skeleton, a hypothesis that is supported by the new finding that VEGF is expressed robustly in the perichondrium and surrounding tissue of cartilage templates of future bones well before blood vessels appear in these regions. We also describe new in vivo evidence for a possible role of VEGF in chondrocyte maturation, and document that VEGF has a direct role in regulating osteoblastic activity based on in vivo evidence and organ culture experiments.

Key words: Mouse, VEGF, Angiogenesis, Blood vessels

INTRODUCTION

Skeletal development in vertebrates is initiated by migration of cells from cranial neural crest, somites and lateral plate mesoderm to the sites of future bones. At these sites (between E10.5 and E12.5 in the mouse), mesenchyme condenses and configures the future skeletal elements. In the cranial vault, jaws and part of the clavicle, the condensed mesenchymal cells differentiate directly into osteoblasts in a process termed intramembranous ossification (Hall and Miyake, 1992; Huang et al., 1997). The remainder of the future skeleton develops by a process known as endochondral bone formation. During endochondral bone formation, the condensing mesenchymal cells differentiate into chondrocytes, which then proliferate and produce extracellular matrix. As development proceeds, chondrocytes in the centers of the cartilage templates (anlagen) cease to proliferate and mature to hypertrophy. 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 (Karsenty, 1999; Olsen et al., 2000).

VEGFA and its receptors VEGFR1 and VEGFR2 are important regulators of angiogenesis during endochondral ossification. Inhibition of VEGF by administration of a soluble chimeric VEGF 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 expansion of the hypertrophic zone (Gerber et al., 1999). Conditional deletion in the mouse of a single VEGFA allele in cells expressing collagen type II 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 was observed (Haigh et al., 2000). Expression of VEGF by hypertrophic chondrocytes requires the expression of the transcription factor Cbfa1; in Cbfa1-deficient mice there is an almost complete lack of VEGF expression in hypertrophic chondrocytes as well as a dramatic decrease in levels of the receptors for VEGF in perichondrial cells (Zelzer et al., 2001).

There is also suggestive evidence from in vitro experiments that VEGF may regulate bone formation through a direct effect on osteoblasts. It has been reported that human VEGF165

binds to osteoblasts in culture and is capable of inducing migration and alkaline phosphatase activity but not proliferation of these cells (Midy and Plouet, 1994). Indeed, it has been shown that VEGFR1 and VEGFR2 are expressed by osteoblasts (Deckers et al., 2000). Not only does VEGF act upon osteoblasts in culture but osteoblasts also produce VEGF when stimulated by vitamin D3 (Wang et al., 1996). Furthermore, VEGF acts as a chemoattractant for osteoclasts (Engsig et al., 2000). These data raise the possibility that VEGF has multiple roles during bone development in vivo. Examination of these roles in vivo is made difficult, however, by the critical function of VEGF in several essential processes during embryonic development; the loss of a single VEGFA allele in mice is sufficient to cause embryonic lethality between E11 and E12 (Carmeliet et al., 1996; Ferrara et al., 1996; Ferrara et al., 1998) as a result of severe cardiac and vascular anomalies; even conditional loss of a single VEGFA allele in type II collagen-expressing cells results in lethality of most embryos around E10.5 (Haigh et al., 2000). Moreover, both VEGFR1- and VEGFR2-null mice die at early stages with defects in the differentiation of hematopoietic and endothelial cells or defective blood vessel formation (Fong et al., 1995; Shalaby et al., 1997; Shalaby et al., 1995).

There are at least three different isoforms of VEGFA in the mouse: VEGF120, VEGF164 and VEGF188. These are products of alternative splicing of a single gene (Ferrara et al., 1992; Shima et al., 1996). VEGF120 does not bind heparan sulfate, suggesting that it is freely diffusible. VEGF164 and VEGF188 possess one and two heparin-binding domains, respectively, allowing interactions with heparan sulfate associated with the cell surface and within the extracellular matrix (Ferrara and Davis-Smyth, 1997; Park et al., 1993). The individual isoforms have been shown to display different binding affinities for VEGFR1 and VEGFR2 (Gitay-Goren et al., 1996; Keyt et al., 1996). Neuropilin 1 is a co-receptor for VEGF164 and can potentiate VEGFR2 activity, but does not bind the VEGF120 isoform (Soker et al., 1998). Recently, mice were produced that expressed only the 120 isoform, and, unlike null heterozygous VEGF mice, these mice survived through embryonic development (Carmeliet et al., 1999). Studies of these $VEGF^{120/120}$ mice revealed impaired myocardial vascularization and pulmonary developmental defects (Carmeliet et al., 1999; Ng et al., 2001), suggesting different activities for the individual isoforms in vivo. Because $VEGF^{120/120}$ mice survive to term, they offer an attractive opportunity to explore the role of VEGF during bone development.

Our study of skeletal development in these mice provide new in vivo evidence for roles of VEGF in normal chondrocyte maturation and regulation of osteoblastic activity. Key differences between $VEGF^{120/120}$ and wild-type mice in vascularization associated with skeletal development, suggest a new role for VEGF in the patterning of skeletal vascularity and cartilage vascularization. The observations are the basis for a two-step model of VEGF-controlled vascularization of skeletal elements.

MATERIALS AND METHODS

Animals

The generation of the VEGF120 isoform-specific mice has been described previously (Carmeliet et al., 1999). In order to generate VEGF120 isoform-specific embryos, $VEGF^{120/+}$ heterozygous male and female mice were crossed to obtain timed-pregnant female mice. The plug date was defined as embryonic day 0.5 (E0.5). For harvesting of embryos, the timed-pregnant female mice were euthanased by exposure to CO_2. The gravid uterus was dissected out and suspended in a bath of cold phosphate buffered saline (PBS) and the fetuses were delivered after amnionectomy and removal of the placenta.

Genotyping

Genomic DNA isolated from portions of the embryos (the tail in E15.5 and older embryos) was used for VEGF genotyping. Genotyping was carried out using PCR with the following three primers: 5′CAG TCT ATT GCC TCC TGA CCT TCA GGG TC3′ (forward primer A, intron 5), 5′CTT GCG TCC ACA CCG TCA CAT TAA GTC AC3′ (reverse primer B, intron 7) and 5′TTC AGA GCG GAG AAA GCA TTT GTT TGT CCA3′ (forward primer C, intron 7), using a standard PCR protocol. The PCR product from the wild-type *Vegf* gene is 400 bp (intron 7 from primers B and C), with 230 bp for the mutant allele (intron 5 to intron 7 from primers A and C, exons 6 and 7 deleted).

Skeletal preparations

Cartilage and bones in whole mouse embryos (E17.5) were visualized after staining with Alcian Blue and Alizarin Red S (Sigma, MO) 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 (7 μm) were stained with Hematoxylin and Eosin (H & E), von Kossa stain and Alizarin Red S, mounted in xylene-based media, and photographed. 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 cut. An automated staining system (BiogenexOptimax Plus, Biogenex, CA) was used. Sections were incubated in order with 100 μg/ml bacterial protease XXIV (Sigma), monoclonal rat anti-mouse CD31 (BD PharMingen, CA) and biotinylated anti-rat IgG. A supersensitive kit (BioGenex, CA) was used for detection. Methyl Green was used for counterstaining.

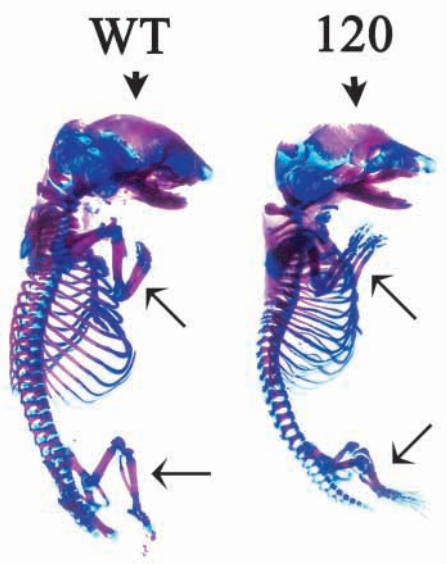

Fig. 1. A comparison of the skeletons of wild-type and $VEGF^{120/120}$ mice reveals reduced size of areas stained for Alizarin Red, suggesting a reduction in mineralization of mutant bones. Regions significantly affected include the long bones in both forelimbs and hind limbs (arrows), and calvarial bones (arrowheads).

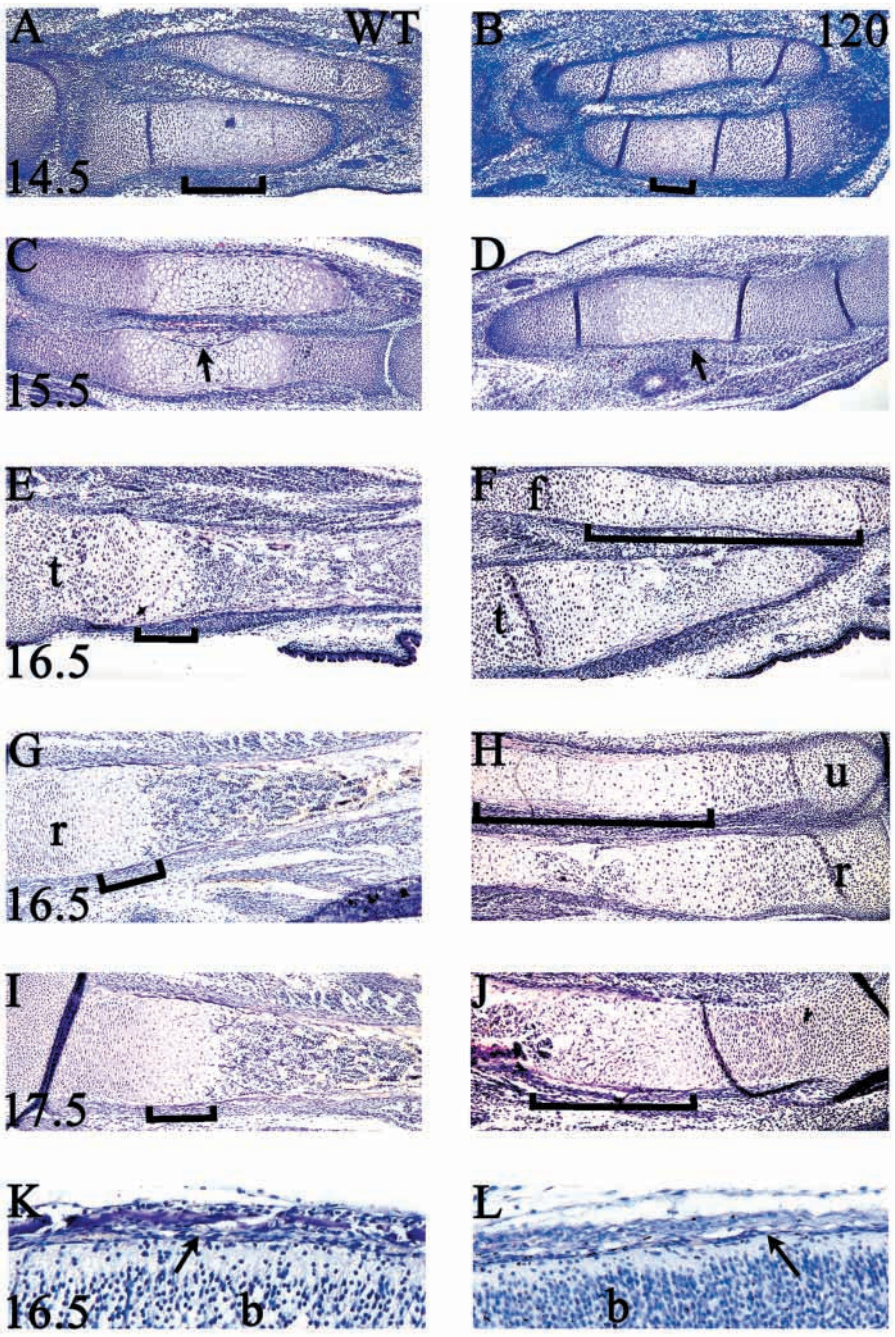

Fig. 2. Histological study of wild-type and VEGF$^{120/120}$ mice identifies marked differences in skeletal elements during development. At E14.5, the hypertrophic zone is larger in wild-type (A) than in VEGF$^{120/120}$ (B) tibia and fibula. At E15.5, blood vessel invasion into the hypertrophic cartilage can be observed in wild-type mice (C, arrow) but not in VEGF$^{120/120}$ mice (D, arrow). (E-H) At E16.5, bone marrow and bone trabeculae are present in wild-type mice (E,G) but there is a significant delay at this stage in VEGF$^{120/120}$ mice (F,H), with blood vessel invasion occurring only in the most central region of the diaphysis. t, tibia; r, radius; f, fibula; u, ulna. (I,J) At E17.5, the tibial VEGF$^{120/120}$ growth plate (J) displays a much longer hypertrophic zone than the wild-type growth plate (I). (K,L) At E16.5, wild-type calvarial bones (K) appear thicker than those in VEGF$^{120/120}$ mice (L) (b, brain; arrow indicates calvarial bone). In Fig. 2A,B,E-J the extent of the hypertrophic zone is indicated by a bracket.

For analysis of osteoclasts, embryonic limbs and heads were fixed in 4% paraformaldehyde and embedded in paraffin for sectioning using standard procedures. Sections (7 μm) were stained for tartrate-resistant acid phosphatase-positive (TRAP$^+$) cells using a TRAP staining kit (Sigma, MO).

In situ hybridization

In situ hybridization was carried out on paraffin sections with ^{33}P-labeled antisense 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 NC1 domain and the 3′-UTR. The VEGFR1 probe consisted of a 1.6 kb cDNA fragment (Finnerty et al., 1993) and the VEGFR2 probe was a 1 kb cDNA fragment (Quinn et al., 1993). The MMP9 probe was a generous gift of Ung-il Chung. The Col2a1 probe consisted of 405 bp from the 3′UTR. The Col1a1 probe was a 183 bp fragment of the carboxyl propeptide domain. The osteocalcin probe was a generous gift from G. Karsenty. The neuropilin 2 probe was kindly supplied by M. Klagsbrun.

β-gal staining

Whole-mount β-gal staining was performed on embryos collected at E13.5-15.5. Embryos were decapitated and limbs removed, prior to fixation in 0.2% glutaraldehyde, 5 mM EGTA pH 7.3, 2 mM $MgCl_2$, 2% paraformaldehyde in 0.1 M sodium phosphate. After washing in 0.1M sodium phosphate pH 8, tissues were incubated in a solution containing 1 mg/ml X-Gal (Sigma, MO), 5 mM K-ferrocyanide and 5 mM K-ferricyanide for 5-16 hours at 37°C. After washing in 0.1 M sodium phosphate pH 7.6, digital images of the stained tissues were made with a SPOT camera (Diagnostic Instruments, MI). Tissues were then infiltrated with 30% sucrose and embedded in Tissue-Tek® OCT. Sections (20 μm) were cut on a cryostat, mounted on Superfrost Plus® slides, dried at room temperature and counterstained with Nuclear Fast Red.

Calvarial cell culture

Calvaria were obtained from E17.5 embryos of Swiss Webster outbred mice (Taconic, NY). Following careful dissection to remove soft tissues, the calvarial bones were washed vigorously in PBS for 20 minutes at room temperature. The bones were then incubated for 20 minutes at 37°C in α-minimum essential medium (α-MEM, Life Technologies, MD) containing 400 units/ml of bacterial collagenase (Type I, Sigma, MO). The medium was discarded and replaced with fresh medium containing collagenase and incubated at 37°C for 30 minutes while shaking. This medium, with released cells, was collected and two more digestions were carried out. Cells from the three digestion steps were collected by centrifugation, suspended in α-MEM containing 10% fetal bovine serum (Paragon Biotech, MD) and glutamine/penicillin/streptomycin (Irvine Scientific, CA), and seeded onto six-well culture plates (Becton Dickinson, NJ) at a

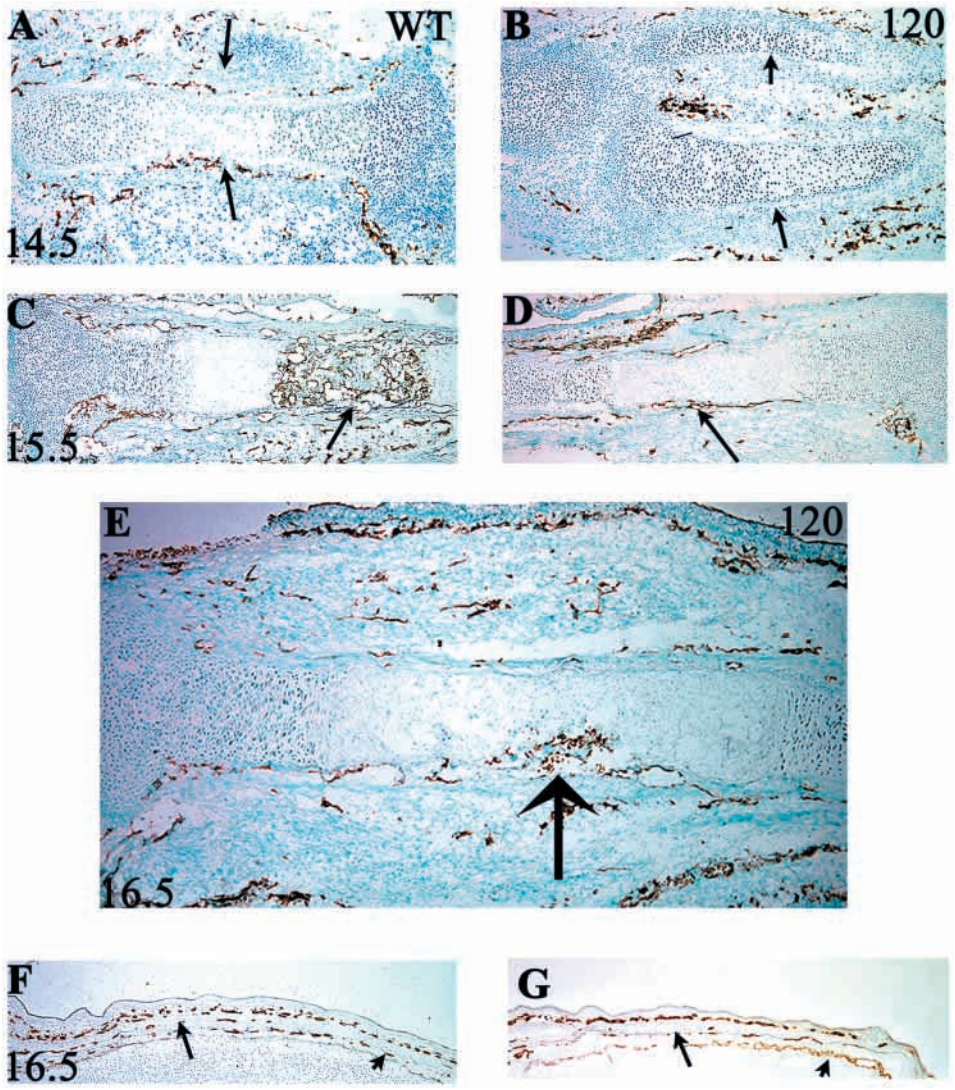

Fig. 3. Comparison of vascularization of wild-type and VEGF$^{120/120}$ skeletal elements by CD31 immunostaining. At E14.5, blood vessels surround the tibia in wild-type mice (A, arrows) but remain far from the surface of the tibia and fibula in the VEGF$^{120/120}$ mice (B; arrows indicate surfaces of tibia and tibula). At E15.5, blood vessels have fully penetrated into the primary ossification center of wild-type (C) tibia (arrow). At E15.5, vessels are only now surrounding the hypertrophic zone in tibia and fibula of VEGF$^{120/120}$ mice (D, arrow), whereas they are invading the diaphysis at E16.5 (E). At E16.5, large numbers of blood vessels surround the developing calvarial bones in the wild-type (F) mice (arrow), but in the VEGF$^{120/120}$ (G) mice there are fewer vessels (arrow), and they appear large in diameter (arrowheads).

concentration of 2.5×10^5 cells per well. Cells were allowed to proliferate to confluence, and daily changes of medium supplemented with 100 μg/ml ascorbic acid (Sigma, MO) and 5 mM glycerophosphate (Sigma, MO) were initiated. After 2-3 weeks, cells were fixed with 4% paraformaldehyde in phosphate buffer pH 7.4 and stained with Alizarin Red S (Sigma, MO). Quantitation of Alizarin Red S-stained material was by analysis of the image of whole wells using the Metamorph (Universal Imaging Corp., PA) image analysis program. Total pixels/well were recorded.

Calvarial explant culture

Calvaria were obtained from E17.5 embryos of Swiss Webster outbred mice (Taconic, NY). After humane sacrifice, the scalps were dissected out and whole calvaria were placed upside down in organ culture dishes (Falcon 3037) on a bed of 1.0% Seakem GTG agarose gel (FMC, ME) in which a shallow depression had been made by placing a glass bead on the agarose surface before the agarose solidified. The use of this depression ensured maintenance of the calvarial shape. The calvaria explants were then covered by a thin layer of 1% agarose. The agarose was dissolved in α-MEM (Life Technologies, MD) containing 10% fetal bovine serum, 100 μg/ml ascorbic acid (Sigma, MO), 5 mM glycerophosphate (Sigma, MO) and antibiotics. The explants were incubated with 1 ml of the medium with 5 ng/ml of rmVEGF164 (R & D Systems, MN), or in medium with 100 ng/ml of Flt-fc (R & D Systems, MN), or in medium without VEGF. Medium was changed daily.

The samples were harvested on day 5, fixed with 4% paraformaldehyde in phosphate buffer pH 7.4 and embedded in OCT compound. Serial coronal sections, which included bilateral parietal bones, were cut at 8 μm thickness. The sections were stained for alkaline phosphatase and mineral (von Kossa), and pictures were taken with a SPOT camera (Diagnostic Instruments, MI).

The thickness of the parietal bone was determined at three defined points of each calvarium, by analysis of images taken of the whole calvaria prior to processing the sample. The points were defined bilaterally as follows. The first two points lay upon a coronal line that goes through the most posterior point of the coronal suture. The first point (P1) and second point (P2) were 1.2 mm and 2.4 mm from the midline, respectively. The third point (P3) lay 1.8 mm from the midline on the section that went through the mid-point of the sagittal suture. The thickness was measured as the distance at a fixed magnification between the inner and outer surfaces of the bone based upon alkaline phosphatase staining. Three calvaria were assessed for each treatment, providing information from a total of 18 points of measurement for each treatment.

To assess bone growth based on these three points of measurement, we normalized the values obtained by comparing each experimental value with the appropriate control. This allowed for combined data from each point in the experimental groups to be used for assessment of the effects of treatments.

RESULTS

VEGF$^{120/120}$ mice exhibit impaired embryonic bone development

To study the role of VEGF in the developing skeleton, we initially examined skeletons of wild-type and VEGF$^{120/120}$ mice at E17.5 using Alizarin Red and Alcian Blue staining. As

can be seen in Fig. 1, the Alizarin Red stained zones are dramatically smaller in the VEGF$^{120/120}$ mice than in the wild-type littermates, suggesting a reduction in mineralization. This reduction is observed in both endochondral bones, such as the long bones in the limb, and in membranous bones, such as the calvarium. For more detailed study, histological sections were prepared of different skeletal elements from stages E14.5 to E17.5. As can be seen in Fig. 2A,B, there is a small size difference between the hypertrophic zones of the wild-type and the VEGF$^{120/120}$ bones at E14.5, while this size difference is less obvious at E15.5 (Fig. 2C,D). At E15.5 in wild-type mice, blood vessel invasion into the hypertrophic cartilage of long distal limb bones (tibia and fibula) has begun (Fig. 2C), but no invasion is observed in the VEGF$^{120/120}$ mice at this stage (Fig. 2D). At E16.5 (Fig. 2E-H), the tibia and radius of wild-type mice contain a well established marrow cavity. By contrast, in the VEGF$^{120/120}$ tibia, fibula, radius and ulna there is an expansion of the hypertrophic zone, which occupies most of the diaphysis. No formation of trabecular bone is observed and blood vessel invasion is present only in the most central region of the diaphysis. At E17.5 (Fig. 2I,J), blood vessels have invaded the cartilage of the tibia in VEGF$^{120/120}$ mice and a marrow cavity is formed, but the zone of hypertrophic chondrocytes (Fig. 2J) remains larger than the zone in wild-type tibia (Fig. 2I).

Intramembranous bones were also affected in the VEGF$^{120/120}$ mice. The calvarial bones were less well developed in the VEGF$^{120/120}$ mice, with a reduction in bone thickness as shown at E16.5 in Fig. 2K,L comparing wild-type with VEGF$^{120/120}$ mice.

Impaired skeletal angiogenesis in VEGF$^{120/120}$ mice

As reported for other organ systems in the VEGF$^{120/120}$ mice (Carmeliet et al., 1999), there appears to be a slight reduction in blood vessel numbers and some increase in vessel diameters in several tissues. Most striking, however, are differences in the pattern of vascularity of skeletal elements in limbs from the VEGF$^{120/120}$ compared with those of wild-type mice.

At E14.5, whereas the wild-type tibia and fibula are surrounded by blood vessels as can be seen by staining for PECAM (CD31) (Fig. 3A,B), blood vessels are not present in the immediate vicinity of the VEGF$^{120/120}$ bones. At E15.5 (Fig. 3C,D) blood vessels have invaded the wild-type tibia and fibula. At this stage, the VEGF$^{120/120}$ tibia and fibula are now surrounded by blood vessels but they have not yet invaded the hypertrophic cartilage. At E16.5 in the wild-type animals, blood vessels have fully invaded the marrow cavity, whereas in the VEGF$^{120/120}$ tibia and fibula, vessels have yet to invade hypertrophic cartilage (Fig. 3E). At E17.5, the diaphyseal regions of tibia and fibula in VEGF$^{120/120}$ mice have been invaded by blood vessels and the marrow cavities have formed (data not shown).

On histological examination of calvaria of VEGF$^{120/120}$ mice, a reduction of vascularity in the regions where the calvarial bones are developing and an increase in the diameter of vessels beneath the calvaria, is evident (Fig. 3F,G).

VEGF expression in hypertrophic chondrocytes of primary ossification centers at E14.5-E15.5 is well documented, but this cannot explain the first appearance of blood vessels in the perichondrium of the cartilages in the developing limb, as this occurs before VEGF expression by hypertrophic chondrocytes.

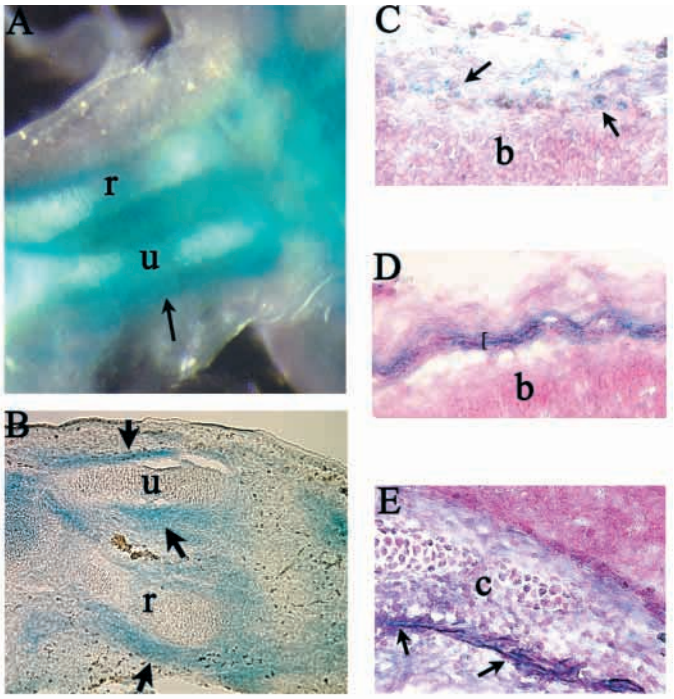

Fig. 4. VEGF-*lacZ* expression in developing limb and calvarium. At E13.5, there is robust expression of VEGF in the periochondrium and surrounding tissues (arrow) of the radius (r) and ulna (u) as observed in whole-mount (A) and histological sections (B). In the calvarium at E13.5, expression of VEGF can be seen (C, arrows; b, brain), and this expression is stronger at E14.5 in the region of mesenchyme that is destined to differentiate into osteoblasts (D, area of expression defined by bracket; b, brain). At E14.5, VEGF expression is associated with cells covering the surface of calvarial bone (E, arrows; c, cranial base cartilage) in regions where osteoid and mineral are being deposited.

VEGF expression at this earlier stage in the developing limb has not previously been characterized. We therefore examined early VEGF expression using mice with an IRES-*lacZ* reporter cassette inserted into the 3′UTR of VEGF (Miquerol et al., 1999).

At E13.5, we observe strong *lacZ* expression in the perichondrium and tissues that surround the radius and ulna, with increased intensity in the area surrounding the center of the cartilage template where the blood vessels will penetrate into the cartilage (Fig. 4A,B). This peripheral expression is reduced at E14.5 and it has disappeared when VEGF expression is seen in the hypertrophic chondrocytes at E15.5 (data not shown). *lacZ* expression is also seen in the developing calvaria where expression patterns change between E13.5 and E15.5. Most striking is the mesenchymal expression of VEGF, at E14.5, in the region that will undergo ossification; at this stage there is high expression in a restricted area (Fig. 4C-E).

The dynamic expression pattern of VEGF in the perichondrium and surrounding tissues at E13.5, and later in hypertrophic chondrocytes at E15.5, coupled with the impaired skeletal angiogenesis in the VEGF$^{120/120}$ mice, suggest that VEGF regulates both the recruitment of the blood vessels to the surface of the cartilage, and the subsequent vascular invasion into the cartilage. Furthermore, it emphasizes the importance of the VEGF164 and VEGF188 isoforms for vascularization of the developing bone.

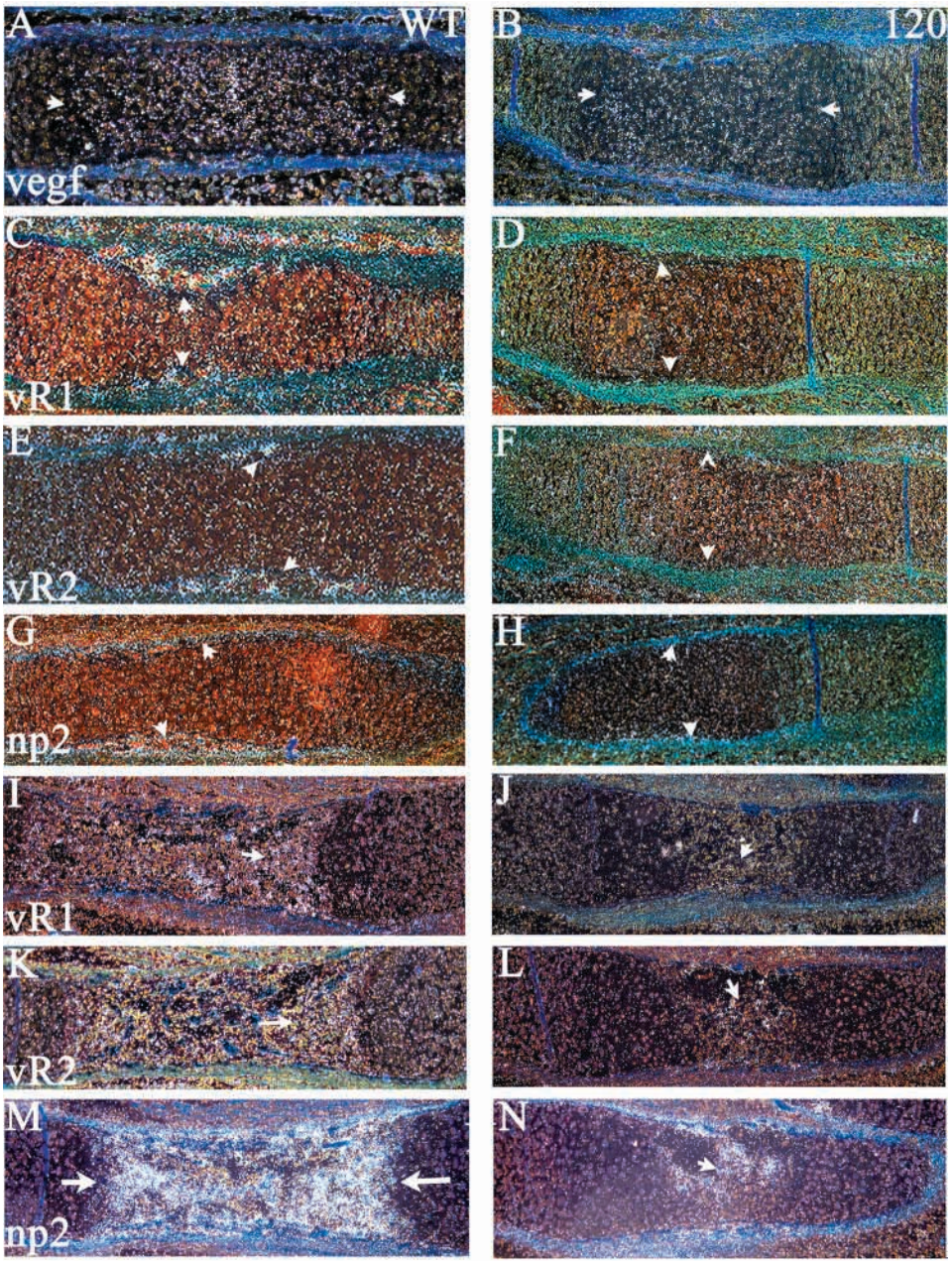

Fig. 5. Expression analysis of VEGF and its receptors, VEGFR1 (vR1), VEGFR2 (vR2) and neuropilin 2 (np2) in the developing tibia. No significant differences are observed in VEGF expression between wild-type (A) and $VEGF^{120/120}$ (B) mice at E15.5 (arrowheads define the regions of VEGF expression). At E15.5, there is significantly higher level of expression of VEGFR1 and VEGFR2 in wild-type tibia (C,E, arrowheads) than in $VEGF^{120/120}$ tibia (D,F, arrowheads). No significant differences are observed in neuropilin 2 expression between wild-type (G) and $VEGF^{120/120}$ (H) mice at E15.5. At E16.5, there is a significantly stronger signal for VEGFR-1 and VEGFR-2 in wild-type tibia (I,K, arrowheads) than in $VEGF^{120/120}$ tibia (J,L, arrowheads). At E16.5, there is a dramatic difference in the expression of neuropilin 2 in wild-type mice (M, arrows) when compared with $VEGF^{120/120}$ mice (N, arrows).

Expression of VEGF and its receptors in $VEGF^{120/120}$ mice

Upregulation in the expression of VEGF in hypertrophic cartilage is believed to be an important signal for the initiation of blood vessel invasion into the hypertrophic zone of cartilage templates of developing bones (Gerber et al., 1999; Zelzer et al., 2001). A possible explanation for the impaired angiogenesis in the $VEGF^{120/120}$ mice is a reduction in the total expression level of VEGF. To examine this possibility, we examined the VEGF expression at E15.5 in wild-type and $VEGF^{120/120}$ mice by in situ hybridization, but failed to observe any differences (Fig. 5A,B). This finding is consistent with results reported by Carmeliet et al. (Carmeliet et al., 1999), in which quantitative RNase protection analysis of VEGF expression in different tissues demonstrated similar expression levels of total VEGF mRNA in $VEGF^{120/120}$ mice and wild-type mice.

The observation that the VEGF expression level in the $VEGF^{120/120}$ tibia and fibula hypertrophic zones appears to be similar to that of the wild-type, suggests that VEGF120 is less ‘effective’ than the VEGF164 and/or VEGF188 in its ability to stimulate the invasion of blood vessels into hypertrophic cartilage. In order to investigate this possibility, we examined the expression of the receptors for VEGF, VEGFR1 and VEGFR2, as upregulation of VEGF expression in the hypertrophic zone leads to upregulation of the expression of the VEGF receptors in the perichondrium. A decrease in VEGF signal leads to a decrease in expression of these two receptors; thus, expression of the VEGF receptors reflects the strength of VEGF induction (Gerber et al., 1999; Zelzer et al., 2001). At E15.5, we observe expression of both VEGFR1 and VEGFR2 on the internal side of the overlying perichondrium/periosteum in the wild-type radius (Fig. 5C,E). This expression is most dramatic at the interface between the perichondrial cells and the terminal hypertrophic chondrocytes. Later, at E16.5, when blood vessels have fully penetrated the cartilage, the expression of the two VEGF receptors is strong in the bone marrow and at the interface between the forming bone and the terminal hypertrophic chondrocytes in the growth plate (Fig. 5I,K). At E15.5, in the $VEGF^{120/120}$ skeleton, we fail to detect strong expression of VEGFR1 and VEGFR2 receptors in the perichondrium (Fig. 5D,F). At E16.5, we observe only weak expression in the center of the diaphysis at the site of initial blood vessel invasion into the cartilage (Fig. 5J,L). It is important to emphasize that

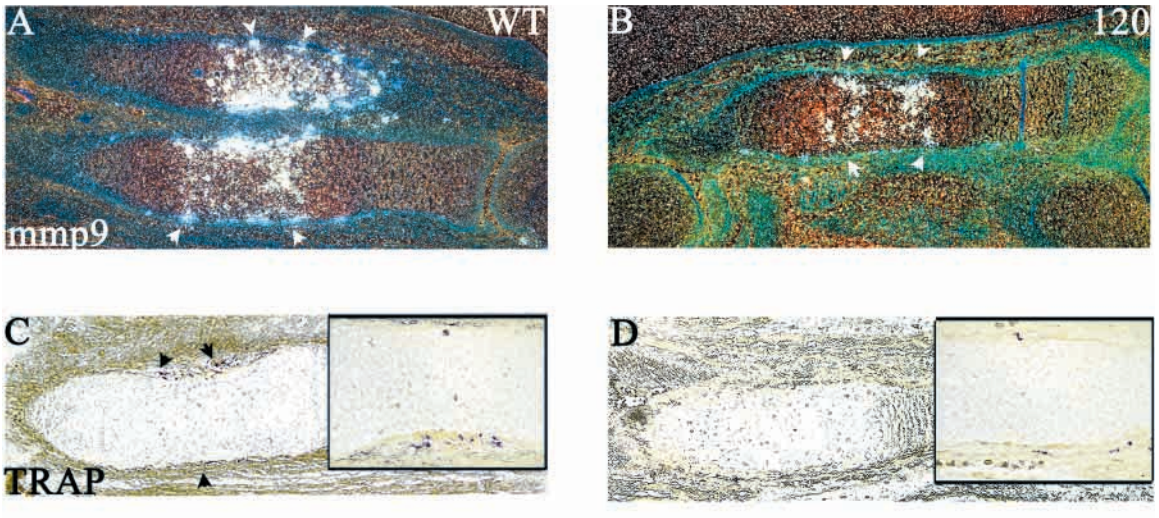

Fig. 6. Analysis of osteoclast markers demonstrates reduced numbers of osteoclasts in the perichondrium of VEGF$^{120/120}$ mice. MMP9 expression in wild-type (A) radius and ulna (arrowheads) is much more extensive than in VEGF$^{120/120}$ (B) radius (arrowheads). This is consistent with the results of TRAP staining. Numerous TRAP-positive cells are seen in the perichondrium of wild-type radius (C, arrowheads), in contrast to the almost complete absence of TRAP-positive cells in VEGF$^{120/120}$ radius (D). Inserts show TRAP-positive cells at a higher magnification.

with the exception of the expression at the interface between the perichondrium and the hypertrophic zone, we did not observe any major differences in expression of VEGFR1 and VEGFR2 receptors in other structures of the limb in wild-type and VEGF$^{120/120}$ mice. At E15.5, we observe little difference in the expression of neuropilin 2 between wild-type and VEGF$^{120/120}$ mice (Fig. 5G,H). This is far from true at E16.5, however, when the expression of neuropilin is massively increased in wild-type mice, but not increased in VEGF$^{120/120}$ mice (Fig. 5M,N). Many cell types have been shown to express this receptor; therefore, the observed difference may not only represent differences of expression in skeletal cells.

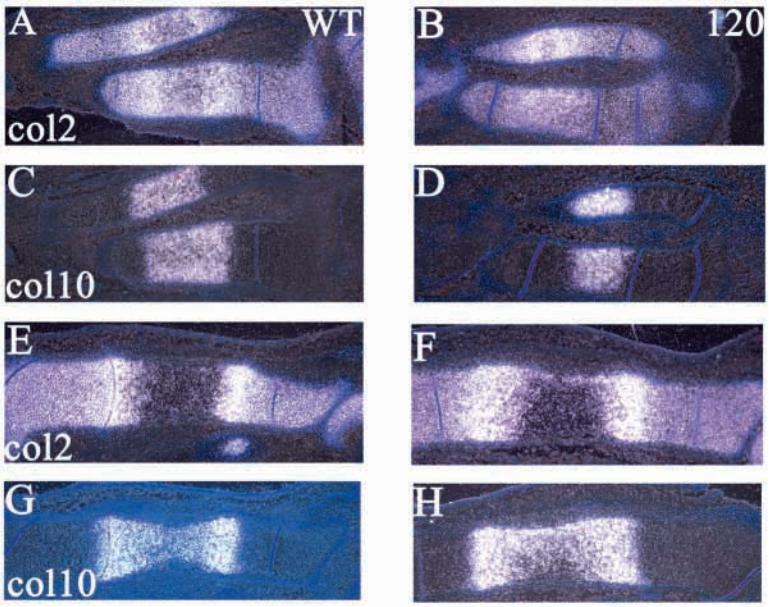

Fig. 7. Expression of chondrocyte differentiation markers. At E14.5, Col2a1 expression is seen throughout the cartilage anlagen in both wild-type (A) and VEGF$^{120/120}$ (B) mice. In the wild-type cartilage there is a region of lower Col2a1 expression in the center of the diaphysis, but this is not as apparent in VEGF$^{120/120}$ mice. In both wild-type (C) and VEGF$^{120/120}$ (D) mice at E14.5, Col10a1 expression is seen in the region of chondrocyte differentiation to hypertrophy. By E15.5, the differences observed between wild-type and VEGF$^{120/120}$ mice in Col2a1 (E,F) and Col10a1 (G,H) expression are small.

Reduced numbers of osteoclasts in the perichondrium of VEGF$^{120/120}$ mice

The invasion of blood vessels into hypertrophic cartilage is coupled to recruitment of chondroclastic/osteoclastic cells into the hypertrophic zone, with VEGF playing a role in both processes (Engsig et al., 2000). Analysis of MMP9 mRNA expression, a marker for chondroclastic/osteoclastic cells, at E15.5, the time when the invasion of blood vessels is initiated in the radius of VEGF$^{120/120}$ and wild-type mice (Fig. 6A,B), reveals that in the wild-type mice, MMP9 is expressed by cells in the hypertrophic region and in the periosteum at the site of the invasion by chondroclasts/osteoclasts. In the VEGF$^{120/120}$ radius, we observe normal expression by cells in the hypertrophic region but a markedly reduced expression in the periosteum. To confirm the apparent reduction in osteoclast numbers, we stained for tartrate-resistant alkaline phosphatase (TRAP), and found a significant reduction in the number of TRAP-positive cells in the VEGF$^{120/120}$ mice (Fig. 6C,D).

Developmental differences in cartilage between VEGF$^{120/120}$ and wild-type mice

The robust early expression of VEGF in the perichondrium (Fig. 4A,B) of wild-type mice and the lack of normal vascularization (Fig. 3B,D,E) in VEGF$^{120/120}$ mice may affect the subsequent

sequence of events in cartilage leading to endochondral ossification. To examine this, we studied the temporal expression of cartilage differentiation markers (Fig. 7).

At E14.5 in wild-type tibia, there is a zone of decreased Col2a1 expression at the center of the cartilage template in the region where chondrocytes undergo hypertrophy. This decreased expression of Col2a1 overlaps with expression of Col10a1 by these cells (Fig. 7A,C). In VEGF$^{120/120}$ mice, downregulation of Col2a1 in the centers of the tibia and fibula is much less apparent, and the area of Col10a1 expression is reduced relative to the area of expression in the wild-type tibia (Fig. 7B,D). At E15.5, the differences in the expression of these markers between wild-type and VEGF$^{120/120}$ mice are small. Col2a1 expression is now reduced in hypertrophic chondrocytes, although, in the VEGF$^{120/120}$ tibia, the zone of hypertrophic chondrocytes that expresses Col2a1 is slightly broader than in wild-type mice. Col10a1 expression in both cases is reduced in the most mature hypertrophic chondrocytes at the center of the hypertrophic zone (Fig. 7E-G,H).

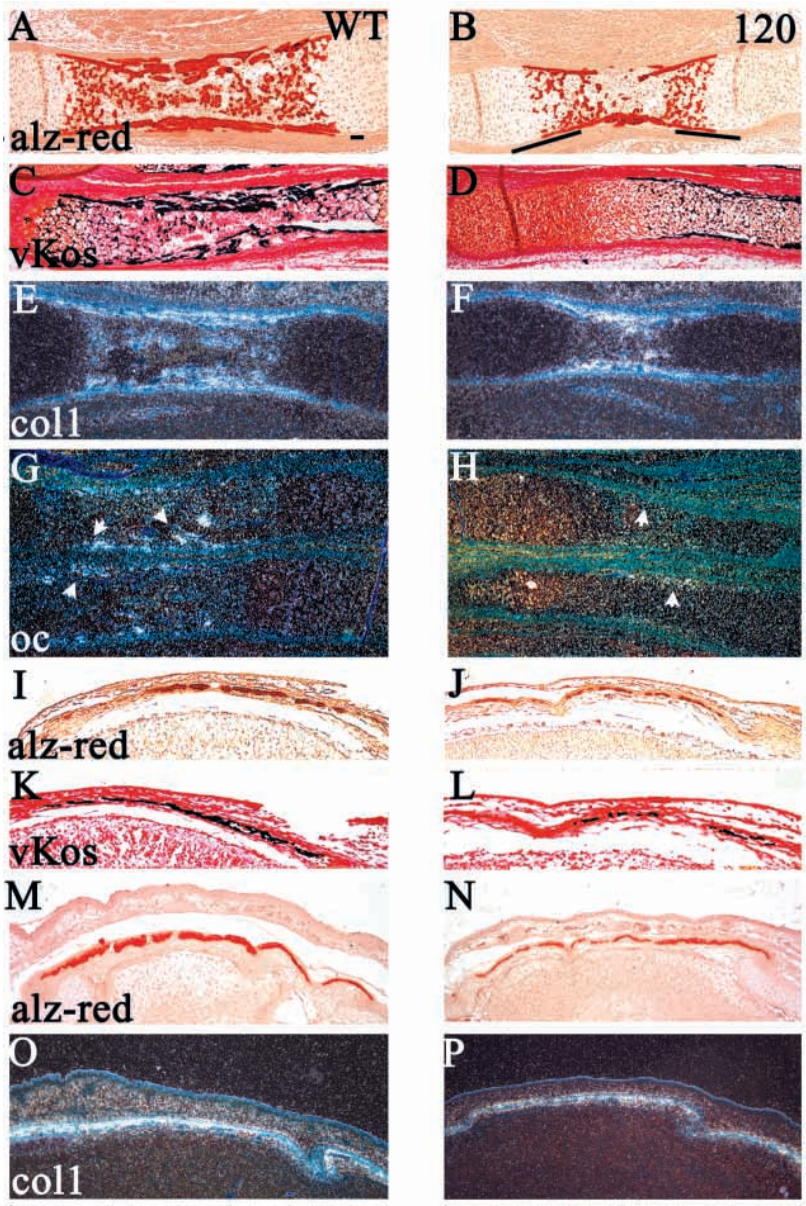

Fig. 8. Comparative analysis of mineralization and expression of osteoblastic markers in long bones and calvaria. At E16.5, there are dramatic differences in the mineralization of wild-type (A, Alizarin Red; C, von Kossa) and VEGF$^{120/120}$ (B, Alizarin Red; D, von Kossa) tibias (A,B) and radii (C,D). Most of the mineralization that is observed in the tibia and radius of VEGF$^{120/120}$ mice is not associated with osteoblastic activity, but is hypertrophic zone mineralization (hypertrophic zone identified by bold line, A and B). At E16.5, expression of Col1a1 in wild-type tibia (E) is much more extensive than in VEGF$^{120/120}$ tibia (F). At E16.5, expression of osteocalcin in wild type (G) is much stronger than that in VEGF$^{120/120}$ radius and ulna (H) (arrowheads identify regions of expression). At both E15.5 and E16.5, reduced mineralization is observed in VEGF$^{120/120}$ mouse calvaria (J,L,N) when compared with wild-type calvaria (I,K,M). At E16.5, expression of Col1a1 is significantly stronger in the wild-type calvarium (O) than in the VEGF$^{120/120}$ calvarium (P).

Reduced osteoblastic activity in VEGF$^{120/120}$ mice

The decreased alizarin red staining of VEGF$^{120/120}$ skeletons (Fig. 1) suggested reduced bone formation in these mice. To determine the involvement of VEGF in the regulation of bone formation, we studied mineralization and markers for osteoblastic differentiation in wild-type and VEGF$^{120/120}$ skeletons. In long bones of the distal limb at E16.5 (Fig. 8A-D), the bone collar is thick and extensive trabeculae are observed by both Alizarin Red and von Kossa staining of wild-type tissue (Fig. 8A,C), whereas in the VEGF$^{120/120}$ bones, the bone collar is thin and fewer trabeculae are observed (Fig. 8B,D). Most of the stained mineral in the VEGF$^{120/120}$ limb is due to mineralization of hypertrophic cartilage. Commensurate with the decreased mineralization of bones in the VEGF$^{120/120}$ mice, we observed a reduction in the expression of osteoblastic markers such as Col1a1 and osteocalcin (Fig. 8E-G,H). At E17.5 (data not shown), there are still significant differences when compared with the wild-type, although blood vessels at this time have invaded the cartilage of the VEGF$^{120/120}$ mice and a marrow cavity with trabecular bone has formed.

The gross histological differences seen between membranous bone of wild-type and VEGF$^{120/120}$ mice (Fig. 2K,L) were further explored by comparing both mineralization and osteoblastic markers in the VEGF$^{120/120}$ calvaria with wild-type calvaria. At E15.5 (Fig. 8I-L) and E16.5 (Fig. 8M,N), we observe that both Alizarin Red and von Kossa staining in the VEGF$^{120/120}$ mice are significantly reduced compared with wild-type mice. In addition, as in long bones, Col1a1 expression is reduced in VEGF$^{120/120}$ mice compared with wild-type mice (Fig. 8O,P).

The involvement of VEGF in angiogenesis and the differences in vascularity described above pose a problem in evaluating a possible direct role for VEGF in osteoblastic differentiation/function during bone development, because an insufficient oxygen and nutrient supply could be implicated in causing reduced bone formation. Therefore, to find

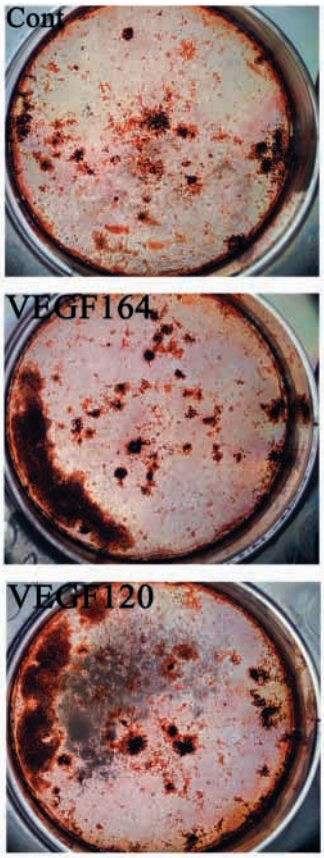

Fig. 9. Mineral deposition in calvarial bone cell cultures. Incubation of primary calvarial cells under conditions that allow mineral deposition in the absence (cont; 20,000 pixels/well) and presence of 25 ng/ml VEGF164 (57,000 pixels/well) or VEGF120 (100,000 pixels/well) shows increased mineralization when recombinant VEGF is present.

out whether VEGF may be a direct regulator of osteoblastic function, we turned to an organ explant and cell culture approach.

VEGF has been shown to have only weak mitogenic activity in osteoblastic culture, and when we examined the ability of two VEGF isoforms (120 and 164) to induce proliferation in primary calvarial cell cultures (using thymidine incorporation) no significant effects were observed (data not shown). Furthermore, we used BrdU labeling to assess periosteal cell proliferation in wild type and VEGF$^{120/120}$ mice. No significant difference was observed (data not shown). To examine the ability of VEGF to affect bone formation, we first tested the ability of the two isoforms to induce mineralization in primary calvarial cell cultures. As shown in Fig. 9, addition of recombinant VEGF120 or VEGF164 to such cultures significantly increased deposition of mineral as measured by staining with Alizarin Red. We also examined the role of VEGF in bone growth in calvarial explants. Explants from E17.5 embryos were cultured for 5 days under conditions that allowed bone growth to continue without distortion of the overall shape of the calvaria. At the end of the culture period, coronal sections were prepared from precisely defined locations (Fig. 10A) and staining of the sections for alkaline phosphatase allowed visualization of the osteoblastic layers on the outside and inside of the calvarial bone (Fig. 10B). Measurements of the distance between the osteoblastic layers at precisely defined points (Fig. 10B) were made on sections from explants that had been cultured without VEGF or with 5 ng/ml of VEGF164 or 100 ng/ml of the chimeric soluble VEGF receptor Flt-fc. In addition, we measured the thickness of calvarial bones at the start of the culture period. As can be seen in Fig. 10C, VEGF164 was able to stimulate a significant thickening of calvarial bones during 5 days of incubation in explant culture.

By contrast, addition of the soluble receptor protein completely abolished the growth of calvarial explants during 5 days in culture. The results clearly demonstrate that thickening of calvarial explants in this model system of bone growth is VEGF dependent. Combined with the impaired membranous

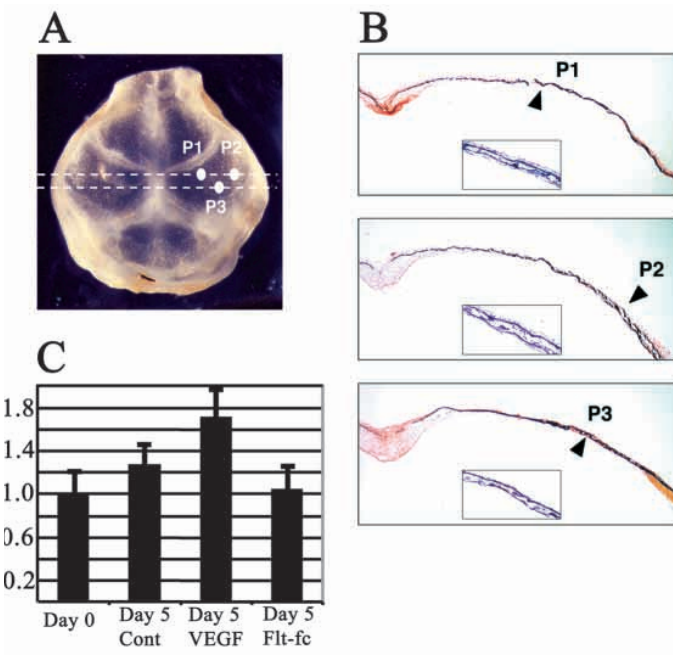

Fig. 10. Calvarial organ culture demonstrates stimulatory effect of VEGF on bone formation. Calvarial explants were cultured without or with VEGF164 or with Flt-fc. The thickness of the parietal bone was measured at three different points (P1, P2 and P3) determined by analysis of images taken of the whole calvaria prior to processing the sample (A). The thickness was measured as a distance between inner and outer surfaces of the bone based upon alkaline phosphatase staining of the osteoblasts (see high magnification insets) (B). VEGF164 treatment led to a significant increase in calvarial bone thickness (average thicknesses determined at points P1, P2 and P3) when compared with the control, while treatment with Flt-fc prevented all growth during the culture period (C).

and endochondral bone formation in the VEGF$^{120/120}$ skeleton, the results provide compelling evidence for a direct role of VEGF in regulating osteoblastic activity.

DISCUSSION

This study identifies several steps at which VEGF has a major regulatory role during the process of bone development. At the same time, because of the well-defined nature of the skeleton as a model for organogenesis, the work contributes to the understanding of the functions of VEGF isoforms in organ development.

The role of VEGF isoforms during bone development

The ability of VEGF$^{120/120}$ mice to proceed through all stages of embryonic development in contrast to the early embryonic lethality of mice that carry one allele of VEGFA, suggests an extensive redundancy between VEGF120 and the two other isoforms VEGF164 and VEGF188. Developmental defects in specific organs and tissues such as heart and lungs in VEGF$^{120/120}$ mice, however, suggest isoform-specific roles as well (Carmeliet et al., 1999; Ng et al., 2001). The precise mechanism of such roles is not clear, but the observations

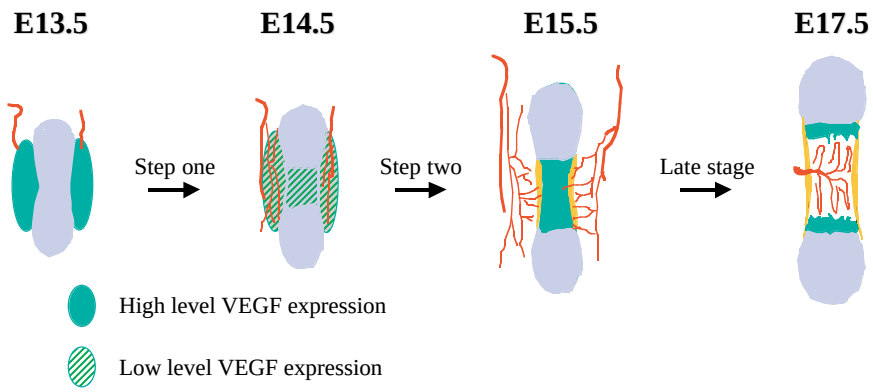

Fig. 11. Two-step model of cartilage vascularization. At E13.5, high levels of VEGF are expressed in the perichondrium and surrounding tissues; this stimulates vascular ingrowth into this area. At E14.5, VEGF expression is reduced in the perichondrial regions as expression in the hypertrophic region begins. At E15.5, high-level expression of VEGF in the hypertrophic zone leads to chondroclast/osteoclast recruitment and vascular invasion. At E17.5, VEGF expression is maintained in the hypertrophic zone after a marrow cavity is established.

reported in this paper suggest that VEGF120 is less effective than the other isoforms in stimulating angiogenesis.

Upregulation of VEGF expression by hypertrophic chondrocytes in primary ossification centers of long bones results in upregulation of its receptors in the adjacent perichondrium. A decrease in VEGF signal leads to a decrease in expression of the receptors (Gerber et al., 1999; Zelzer et al., 2001). The correlation between VEGF expression in hypertrophic cartilage and the vascularization of primary ossification centers suggests that the levels of expression of the VEGF receptors reflects the strength of VEGF as an inducer of long bone angiogenesis. Stimulation of endothelial cells by VEGF has been demonstrated to induce migration, proliferation and expression of VEGF receptors (Barleon et al., 1997; Shen et al., 1998). The low expression level of VEGF receptors around primary ossification centers in $VEGF^{120/120}$ mice (Fig. 5D,F) suggests that the 120 isoform is less effective than the other isoforms in regulating these endothelial cell activities. Extensive in vitro studies suggest that the VEGF120 isoform lacks the heparin-binding domain of the isoforms VEGF164 and VEGF188. It is therefore considered a more diffusible form (Neufeld et al., 1999; Robinson and Stringer, 2001). Thus, although the total level of VEGF transcripts may well be the same in $VEGF^{120/120}$ and wild-type mice, local tissue concentrations of VEGF protein may be higher in the wild-type because of the presence of the matrix-binding isoforms. Unfortunately, the lack of appropriate antibodies makes direct measurements of tissue levels of VEGF isoforms not possible; clearly, better antibodies or other methods to distinguish between the different isoforms proteins are essential before this possibility can be examined. Whatever the precise reasons are for the skeletal abnormalities in the $VEGF^{120/120}$ mice, however, the defects represent genetically a hypomorphic phenotype caused by an alteration of VEGF function.

The role of VEGF in establishing cartilage and bone vascularity

At E14.5, the cartilage primordia of the wild-type mouse endochondral skeleton are surrounded by blood vessels (Fig. 3A). Regulation of angiogenesis in these pericartilaginous regions is probably controlled by a process that is independent of VEGF expression by hypertrophic chondrocytes. The evidence presented here suggests that the expression of VEGF by hypertrophic chondrocytes is not sufficient for normal perichondrial and cartilage vascularization. At E14.5, when VEGF expression in the hypertrophic zone is only in its initial phase (Colnot and Helms, 2001), blood vessels are already surrounding the cartilage (Fig. 3A), suggesting that recruitment of vessels into the perichondrium has occurred even before the hypertrophic chondrocytes express VEGF. Another line of evidence comes from previous studies of Cbfa1-null mice. We have demonstrated that VEGF expression is not upregulated in hypertrophic cartilage of tibia in Cbfa1-null mice and yet we still observe an extensive blood vessel network surrounding the tibia (Zelzer et al., 2001).

The expression of VEGF in the limb prior to ossification shows robust expression of VEGF in the perichondrium and surrounding tissues at E13.5 (Fig. 4A,B). This expression identifies a possible source of VEGF for the recruitment of the vascular network into regions surrounding the cartilage prior to chondrocyte hypertrophy. The lack of perichondrial vascularity that we observe at E14.5 (Fig. 3B) in the $VEGF^{120/120}$ limbs suggests that the function of VEGF expressed by perichondrial cells at E13.5, in fact, is to stimulate perichondrial angiogenesis. The model that we propose for vascularization of cartilage is consequently a two-step model (Fig. 11). In the first step, expression of VEGF in the perichondrium at E13.5 attracts blood vessels to the tissue around the developing cartilage. The expression of VEGF in the central perichondrial regions of the cartilage templates is relatively strong, corresponding to the region where invasion of blood vessels into the hypertrophic zone will take place later. At E14.5, the expression of VEGF in this perichondrial region decreases, as the cartilage is now already surrounded by blood vessels. Simultaneously, there is an induction of VEGF expression in the hypertrophic zone. At E15.5, the second step is initiated as cells in the hypertrophic zone express higher levels of VEGF. At the same time, there is chondroclast/osteoclast recruitment and vessel invasion into the cartilage. A dynamic expression pattern of VEGF is also found in the developing calvaria (Fig. 4C-E), where there are marked

differences in expression at E13.5 and E15.5. Most striking is the strong mesenchymal expression of VEGF at E14.5 in the region that will undergo ossification (Fig. 4D). This expression may be essential for the development of the vasculature associated with calvarial bone development.

A possible role for VEGF in chondrocyte maturation

It has been reported that in VEGF heterozygous embryos there is, among other defects, a failure of forelimb bud development (Ferrara et al., 1996). In the $VEGF^{120/120}$ mice, we did not see any disruption in early patterning of the limb. Instead, we observed differences in the extent of expression of cartilage differentiation markers at E14.5 between wild-type and $VEGF^{120/120}$ mice (Fig. 7A-D), suggesting a role for VEGF in cartilage maturation. These changes may arise as a result of altered VEGF signaling at several stages of limb development: early during limb bud formation, at E13.5 when VEGF is expressed in the perichondrium of the distal limb long bones, or at E14.5, resulting from altered expression in the hypertrophic chondrocytes. It has been shown previously that hypertrophic chondrocytes in culture express VEGFR2, which was found to be phosphorylated, suggesting the existence of an autocrine loop for VEGF signaling in hypertrophic chondrocytes (Carlevaro et al., 2000). The exact role of VEGF in chondrocyte maturation still remains unclear, but our observations add to a growing body of evidence supporting a role for VEGF in the process of chondrocyte maturation.

A role for VEGF in control of osteoblastic activity

Endochondral bone formation has, for a long time, been known to be dependent on cartilage vascularization (Trueta and Amato, 1960). Yet, several lines of evidence suggest that VEGF also has a direct effect on the activity of osteoblasts. At E13.5, VEGF is expressed in the perichondrium and surrounding mesenchyme of the long bones of the distal limb and in the skull mesenchyme (Fig. 4). At E14.5, the expression in the skull mesenchyme is stronger and more restricted to the region where mesenchymal cells are differentiating into osteoblasts (Fig. 4). This correlation between VEGF expression and tissues that participate in osteoblast formation is suggestive of a link between the two. Midy and Plouet (Midy and Plouet, 1994) have previously demonstrated that human recombinant VEGF165 is capable of binding to osteoblasts and inducing migration, stimulating PTH-dependent cAMP accumulation, and increasing alkaline phosphatase activity. Indeed, it has been shown that both VEGFR1 and VEGFR2 are expressed by osteoblasts (Deckers et al., 2000). Our observation that VEGF stimulates mineral deposition in calvarial bone cell cultures strengthens the connection between VEGF and bone formation. Furthermore, both intramembranous and endochondral bone formation are disrupted in $VEGF^{120/120}$ mice (Figs 1, 8). Finally, in calvarial explant culture, we demonstrate that VEGF is a regulator of bone formation (Fig. 10). All of the above strongly support a direct role for VEGF in regulating osteoblastic activities.

Conclusions

On the basis of the results presented here, we suggest that VEGF controls at least three aspects of bone development. First, it induces angiogenesis in regions of intramembranous bone formation and in the perichondrial regions of cartilage templates in the endochondral skeleton. Second, it stimulates angiogenesis and chemotactic migration of osteoclastic cells into hypertrophic cartilage. Finally, it stimulates bone formation by increasing the activity of osteoblasts both in intramembranous and endochondral bones.

We are grateful to Drs B. Cohen, U. Chung, G. Karsenty and M. Klagsbrun for providing the probes for VEGF, MMP9, osteocalcin and neuropilin 2, respectively. The VEGF-*lacZ* mice were a gift from Dr A. Nagy. We thank Drs R. Mamluk and D. J. Glotzer for invaluable advice and constructive criticisms throughout the work and during manuscript preparation, Ms Y. Pittel for patient and expert editorial assistance, and members of the Olsen laboratory for continuous advice and suggestions. This work was supported in part by EMBO (to E. Z.), NIH grants AR36819 and AR36820 (to B. R. O.), NIH grant CA45548 (to P. D'A.) and a Research Training Fellowship (S/F/0732) from Action Research (to W. M.).

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