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# The forming limb skeleton serves as a signaling center for limb vasculature patterning via regulation of *Vegf*

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Limb development constitutes a central model for the study of tissue and organ patterning; yet, the mechanisms that regulate the patterning of limb vasculature have been left understudied. Vascular patterning in the forming limb is tightly regulated in order to ensure sufficient gas exchange and nutrient supply to the developing organ. Once skeletogenesis is initiated, limb vasculature undergoes two seemingly opposing processes: vessel regression from regions that undergo mesenchymal condensation; and vessel morphogenesis. During the latter, vessels that surround the condensations undergo an extensive rearrangement, forming a stereotypical enriched network that is segregated from the skeleton. In this study, we provide evidence for the centrality of the condensing mesenchyme of the forming skeleton in regulating limb vascular patterning. Both *Vegf* loss- and gain-of-function experiments in limb bud mesenchyme firmly established VEGF as the signal by which the condensing mesenchyme regulates the vasculature. Normal vasculature observed in limbs where VEGF receptors *Flt1*, *Flk1*, *Nrp1* and *Nrp2* were blocked in limb bud mesenchyme suggested that VEGF, which is secreted by the condensing mesenchyme, regulates limb vasculature via a direct long-range mechanism. Finally, we provide evidence for the involvement of SOX9 in the regulation of *Vegf* expression in the condensing mesenchyme. This study establishes *Vegf* expression in the condensing mesenchyme as the mechanism by which the skeleton patterns limb vasculature.

**KEY WORDS: Skeleton, Skeletogenesis, Anti-angiogenic, Vascular patterning, Limb development, SOX9, VEGF, PRX1-Cre, SOX9-Cre, Mouse**

## INTRODUCTION

The vasculature is one of the first systems to emerge in the embryo, as its functionality is necessary for further development. New blood vessels are formed by two main processes termed vasculogenesis and angiogenesis. Vasculogenesis is characterized by aggregation of angioblasts to form, de novo, a primitive vascular plexus, whereas during angiogenesis, the vascular plexus is expanded through growth, migration, sprouting and pruning of existing vessels (Coffin and Poole, 1988; Drake et al., 1998; Folkman, 2003; Risau and Flamme, 1995; Sabin, 1920; Sato and Loughna, 2002).

One of the key players in both angiogenesis and vasculogenesis is vascular endothelial growth factor (VEGF) (Carmeliet et al., 1996; Ferrara et al., 1996). VEGF controls blood vessel development by regulation of endothelial cell proliferation, migration and differentiation (Brown et al., 1997; Ferrara and Henzel, 1989; Leung et al., 1989). During angiogenesis, VEGF binds to two tyrosine-kinase receptors, VEGFR1 (FLT1) and VEGFR2 (FLK1), which are present predominantly on endothelial cells (Carmeliet and Collen, 1999; de Vries et al., 1992; Fong et al., 1995; Shalaby et al., 1997; Shalaby et al., 1995; Terman et al., 1992). In addition, endothelial cells express the co-receptors neuropilin 1 (NRP1) and neuropilin 2 (NRP2), which bind to VEGF and potentiate FLK1 activity (Neufeld et al., 1999; Soker et al., 1998).

During embryogenesis, one of the challenging tasks the forming organ is faced with is the need to synchronize its development with that of the vasculature, in order to ensure sufficient gas exchange and nutrient supply (Cleaver and Melton, 2003; Coultas et al., 2005; Hogan et al., 2004) Limb development constitutes a central model for the study of tissue and organ patterning (Cohn and Tickle, 1996; Johnson and Tabin, 1997). Up until now, most of the patterning mechanisms that have been extensively studied were related to limb skeleton. Interestingly, and in contrast to its absolute necessity, the mechanisms that regulate the patterning of limb vasculature have been left understudied.

During the initial stages of limb formation, angiogenesis is initiated as sprouts from the dorsal aorta invade the limb bud and form a vascular plexus, which is embedded within the limb mesenchymal core (Seichert and Rychter, 1972a; Seichert and Rychter, 1972b). Concomitantly, vasculogenesis contributes to the forming vascular plexus, as somite-derived angioblasts migrate and integrate into the developing plexus (Ambler et al., 2001). Next, as skeletogenesis is initiated, the initially unpatterned vascular plexus undergoes major spatial changes that result in its rearrangement into a highly branched and patterned network, which is segregated from the forming skeleton. Most prominently, avascularized areas emerge from previously vascularized regions as a result of vessel regression from the emerging cartilage anlage. Concurrently, the surrounding vasculature undergoes an extensive morphogenesis, forming a stereotypical, highly branched and enriched network (Feinberg et al., 1986; Hall and Miyake, 1992; Seichert and Rychter, 1972a). The mesenchymal cells that occupy these avascular areas aggregate and form high cell density condensations that will eventually differentiate into chondrocytes, thus forming cartilage models of the future bones (Hall and Miyake, 2000). Mesenchymal condensation is the initial step in skeleton formation and the transcription factor SOX9 is an essential regulator of this process (Bi et al., 1999). *Sox9* is first expressed in the limb bud between E10 and E10.5 in chondroprogenitors and chondrocytes, preceding the formation of cartilage (Wright et al., 1995). Inactivation of *Sox9* in limb mesenchymal and neural crest cells results in complete absence of mesenchymal condensation and subsequent failure in cartilage formation (Akiyama et al., 2002; Mori-Akiyama et al., 2003).

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The tight coordination between skeleton development and vascular rearrangement has prompted studies that aimed to expose the regulatory role that these two systems were presumed to play on each other's development. Some of these studies addressed the obvious issue of which system is patterned first, assuming that the first system to be patterned may regulate the patterning of the other tissue (Feinberg et al., 1986; Hallmann et al., 1987; Wilson, 1986). Other studies concentrated on the influence of an abnormal vasculature on limb skeleton formation and the mechanism that underlies such an effect (Caplan and Koutroupas, 1973; Feinberg and Saunders, 1982; Fraser and Travill, 1978; Hootnick et al., 1980; Jargiello and Caplan, 1983). However, although these studies have provided strong indications for the possible regulatory interactions between limb vasculature and the skeleton, they were not conclusive, mostly owing to the absence of genetic tools. Thus, the mechanism that coordinates vascular patterning and skeletogenesis remained unsolved.

In this study, we provide evidence for the centrality of the forming skeleton in regulating limb vascular patterning and implicate *Vegf* expression by condensed mesenchyme as a key component in the underlying mechanism. Blocking the expression of the VEGF receptors *Flt1*, *Flk1*, *Nrp1* and *Nrp2* in limb mesenchyme resulted in no apparent effect on vascular patterning, strongly suggesting that VEGF regulates limb vasculature via a long-range mechanism. Finally, we provide evidence for the involvement of SOX9 in the regulation of *Vegf* expression in the condensing mesenchyme. These findings establish *Vegf* expression in the condensing mesenchyme as the mechanism by which the skeleton patterns limb vasculature.

## MATERIALS AND METHODS

### Animals

The generation of *floxed-Vegf* (Gerber et al., 1999), *floxed-Sox9* (Akiyama et al., 2002), *Vegf-IRES-lacZ* (Miquerol et al., 1999), *Sox9-Cre* (Akiyama et al., 2005), *Prx1* (also known as *Prrx1* – Mouse Genome Informatics) -*Cre* (Logan et al., 2002), *rtTA* (Belteki et al., 2005), *tetO-Vegf* (Benjamin and Keshet, 1997), *Nrp2-LacZ* (Chen et al., 2000), *floxed-Nrp1* (Gu et al., 2003), *Nrp2* (Giger et al., 2000) and *Sox9* misexpressed (Akiyama et al., 2007) mice have been described previously; *floxed-Flt1* and *floxed-Flk1* will be described elsewhere. In all timed pregnancies, the day of the vaginal plug appearance was defined as E0.5. For harvesting of embryos, timed-pregnant female mice were sacrificed by $CO_2$ intoxication. The gravid uterus was dissected out and suspended in a bath of cold PBS, and the embryos were harvested after amnionectomy and removal of the placenta. Tail genomic DNA was used for genotyping. All experiments were performed with at least six different control and knockout forelimbs from three different litters.

### Whole-mount and section immunofluorescence and in-situ hybridization

For whole-mount immunofluorescence, freshly dissected tissue was fixed overnight in 4% PFA, transferred to PBS, then dehydrated to methanol and stored in –20°C until use. Samples were rehydrated to PBS and incubated for 2 hours in blocking solution (PBS containing 10% normal goat serum and 1% Triton X-100) and then incubated overnight at 4°C with primary antibody rat anti-PECAM (CD31; BD Pharmingen, San Diego, CA) 1:25 diluted in blocking solution. Samples were washed in PBS containing 1% Triton X-100 at room temperature and then incubated overnight at 4°C with biotinylated anti-rat secondary antibody (dilution 1:100; Vector Laboratories) and Cy2-conjugated streptavidin (1:100; Jackson ImmunoResearch, West Grove, PA) antibodies diluted in 1% BSA/PBS.

Immunofluorescence of cryosections was preformed as described previously (Amarilio et al., 2007). Slides were incubated with the primary antibodies: rat anti-CD31 (BD PharMingen; 1:100), monoclonal anti-collagen type IIa1 (Developmental Studies Hybridoma Bank, The University of Iowa, IA; 1:100), goat anti-rat neuropilin 1 (R&D, Minneapolis, MN; 1:100), rat anti-RAFL-1 (60 μg/ml) and biotin-labeled peanut agglutinin (PNA, Sigma-Aldrich, St Louis, MO; 1:100). Secondary antibodies used were: Alexa Fluor 488-labeled goat anti-rat IgG, Alexa Fluor 568-labeled goat anti-mouse IgG (Molecular Probes), goat anti-rabbit indocarbocyanine (Cy3), goat anti-mouse Cy2, Cy2-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA; 1:100). VEGF (Calbiochem) and CD34 (Abcam) staining was preformed using paraffin sections according to the manufacturer's protocol. Samples were washed, mounted on glass slides and analyzed with a LSM510 laser-scanning confocal microscope (Carl Zeiss, Jena, Germany).

Section in situ hybridization process was preformed as described previously (Murtaugh et al., 1999; Riddle et al., 1993). All probes are available by request.

### X-gal staining

Freshly dissected tissue was fixed in 4% PFA/PBS, rinsed in a solution containing 5 mM EGTA, 0.01% deoxycholate, 0.02% NP40 and 2 mM $MgCl_2$, and then stained in a solution containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 5 mM EGTA, 0.01% deoxycholate, 0.02% NP40, 2 mM $MgCl_2$ and 1 mg/ml X-gal. The tissue was either cleared in 0.3% KOH or dehydrated and embedded in paraffin for longitudinal sections.

### Overexpression of *Vegf* in the condensed mesenchyme

Inducible *Vegf* overexpression in the condensed mesenchyme was carried out by the reverse tetracycline transactivator (rtTA)/tetracycline-responsive element (tetO)-driven transgene system (Belteki et al., 2005; Gossen et al., 1995), with *Sox9-Cre* as an inducer (Akiyama et al., 2005). Briefly, *tetO-Vegf* mice were crossed with *rtTA* mice. Mice heterozygous for *rtTA* and *tetO-Vegf* (*rtTA- tetO-Vegf*) were crossed with mice heterozygous for *Sox9-Cre* transgene as an inducer. To induce *Vegf* expression, doxycycline was administered to pregnant females starting at E10.5 and embryos heterozygous for *Sox9-Cre*, *rtTA* and *tetO-Vegf* (*Sox9-rtTA-tetO-Vegf*) were compared with embryos heterozygous for *rtTA* and *Sox9-Cre* alleles (control).

### Conditional blockage of *Vegf* in limb mesenchyme

Conditional blockage of *Vegf* in limb mesenchyme was obtained by crossing *floxed-Vegf* mice with the *Prx1-Cre* transgenic mouse as a deletor (Logan et al., 2002). Embryos homozygous for *floxed-Vegf* and heterozygous for *Prx1-Cre* alleles *(Prx1-Vegf)* were compared with embryos heterozygous for *floxed-Vegf* and *Prx1-Cre* alleles (control).

### Primary cell culture preparations and viral transfer

For micromass cultures, limbs of E11.0-E11.5 *floxed-Sox9* embryos were collected, digested with 0.1% collagenase IV, 0.1% trypsin (Sigma) and 2% FCS for 15 minutes. The cell suspension was placed in DMEM-F12, 10% FCS. Cells were plated as 10 μl droplets at $2\times10^7$ cells/ml. Cells were allowed to attach for 75 minutes and were then overlaid with 300 μl of DMEM-F12, 10% FCS containing $6.5\times10^7$ viral particles/μl of Adeno-Cre and Ad-βgal (Gene Transfer Vector Core, University of Iowa, IA). Medium was changed daily. Cells were cultured with 20% oxygen in a humidified atmosphere and then harvested to extract RNA.

### Quantitative RT-PCR (qRT-PCR)

For qRT-PCR analysis, 1 μg total RNA was used to produce first-strand cDNA. Reverse transcription was performed with SuperScriptII (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. qRT-PCR was performed using SYBR Green (Roche). Values were calculated using the second derivative method and normalized to 18S rRNA expression. All primers are available on request.

## RESULTS

### Vascular and skeletal development are coordinated during limb organogenesis

Previous studies performed mostly on chick embryos have identified a tight coordination between vascular patterning and skeletal development. In order to study the mechanism that underlies this coordination in a genetically tractable model system, we first

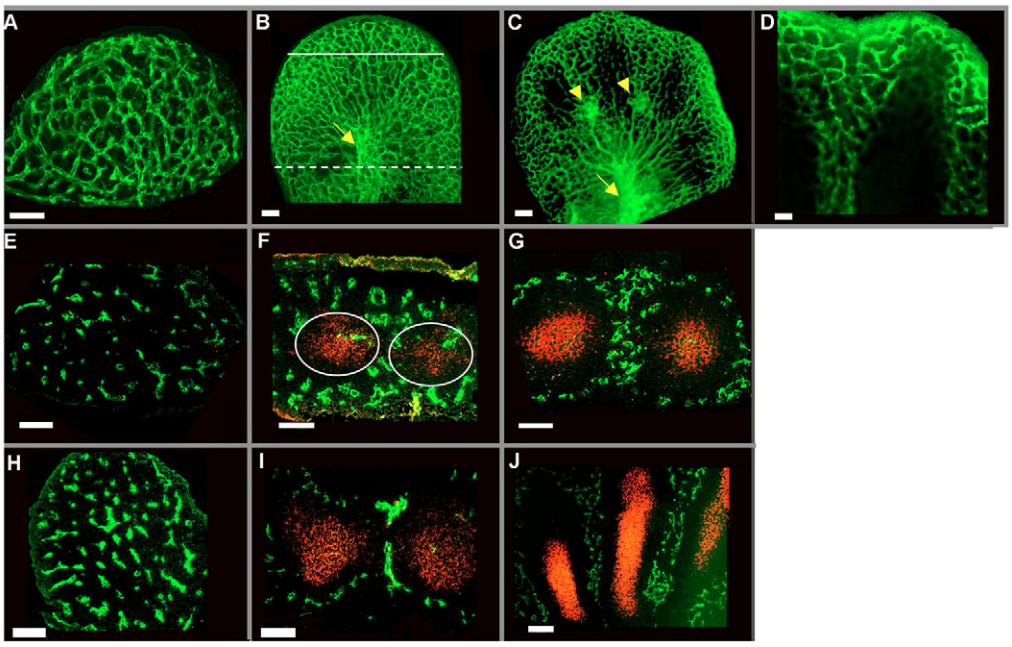


**Fig. 1. Vascular and skeletal developments are coordinated.** (**A-D**) Immunofluorescence staining of wild-type embryos with anti-CD31 (green) shows vascular distribution of E10.5 (A), E11.5 (B) and E12.5 (C) whole limbs and interdigital vasculature near the avascular area of future metacarpal (D). Yellow arrows indicate axial arteries; yellow arrowheads indicate vascular-rich stems that divide future metacarpals; white line indicates area of autopod; broken white line indicates area of radius and ulna. (**E-J**) Immunofluorescence staining with anti-CD31 (vascular endothelial cells, green) and anti-collagen II (chondrocytes, red) antibodies illustrates vascular patterning and chondrocyte differentiation, respectively. (E,H) Vascular patterning in transverse (E) and longitudinal (H) sections of the limb bud at E10.5. (F,I) E11.5 transverse sections of autopod (F) and zeugopod (I) areas, as indicated by unbroken and broken lines in B. Circled areas in F contain mesenchymal cells that undergo differentiation into chondrocytes. (G,J) Transverse (G) and longitudinal (J) sections of the autopod at E12.5. Scale bars: 100 μm.

documented vascular patterning and skeletal development in wild-type mice. To study limb vascular patterning, we stained E10.5-E12.5 whole limbs for endothelial cells using antibodies for PECAM (CD31). At E10.5, the vasculature was comprised of a uniform capillary network of small tubes that were embedded throughout the limb bud (Fig. 1A). By E11.5, the capillary network became denser and a single axial artery that split into the capillary network could be observed (Fig. 1B). One day later, at E12.5, dramatic changes in vascular patterning were clearly visible. In the distal region of the limb (autopod), the axial artery was thicker and split into vascular-rich stems that divided the regions where the metacarpals were forming (Fig. 1C). In these regions, no vessels could be observed (Fig. 1D); instead, they were flanked dorsally and ventrally by a capillary network that originated from the vascular trunks located in the interdigital zones, where a highly dense network of capillaries was formed.

To study the coordination between vascular patterning and skeleton formation, we double-stained longitudinal and transverse sections of E10.5-E12.5 limbs for endothelial cells and chondrocytes, using antibodies for CD31 and collagen type II, respectively. At E10.5, the vasculature was evenly distributed throughout the mesenchymal core of the limb bud (Fig. 1E,H). By E11.5, in the medial region of the limb where the radius and ulna were forming (zeugopod), pre-chondrogenic mesenchymal cells formed condensations and differentiated into chondrocytes. These areas were avascular, whereas in the surrounding vicinity segregated vessels could be observed (Fig. 1I). However, more distally in the autopod, vessels could still be observed in regions where mesenchymal cells were undergoing condensation (Fig. 1F). As development proceeded, at E12.5, limb vasculature was further patterned to become completely segregated from the forming skeleton (Fig. 1G,J). The formation of avascular areas in the autopod was accompanied by an increase in vessel number in the interdigital zones and in regions that surrounded the condensations. These results demonstrate that during mouse limb development, vascular patterning is tightly coordinated with mesenchymal condensation and skeleton formation.

## Skeleton formation is necessary for limb vascular patterning

The discovery of a well-orchestrated process of vascular and skeletal patterning during limb development raised the hypothesis that the forming skeleton regulated the patterning of limb vasculature. The transcription factor SOX9 has been shown to be a key mediator of limb skeletal development (Akiyama et al., 2002; Mori-Akiyama et al., 2003). To uncover the involvement of mesenchymal condensation in the regulation of vascular patterning, we blocked *Sox9* expression in limb mesenchymal cells using the *Prx1-Cre* mouse as a deletor (Logan et al., 2002) and examined the vasculature. Embryos homozygous for *floxed-Sox9* and heterozygous for *Prx1-Cre* alleles (*Prx1-Sox9*) were compared with embryos heterozygous for *floxed-Sox9* and *Prx1-Cre* alleles (control). qRT-PCR of E12.5 control and *Prx1-Sox9* limbs demonstrate 60% decrease in *Sox9* mRNA expression in *Prx1-Sox9* limbs, relative to the control.

Examination of whole limbs and sections of E10.5-E12.5 *Prx1-Sox9* revealed a failure of the vasculature to pattern normally (Fig. 2). At E10.5, the vasculature of both control and *Prx1-Sox9* limbs were comparable (Fig. 2A,B). However, at E11.5 the axial artery that

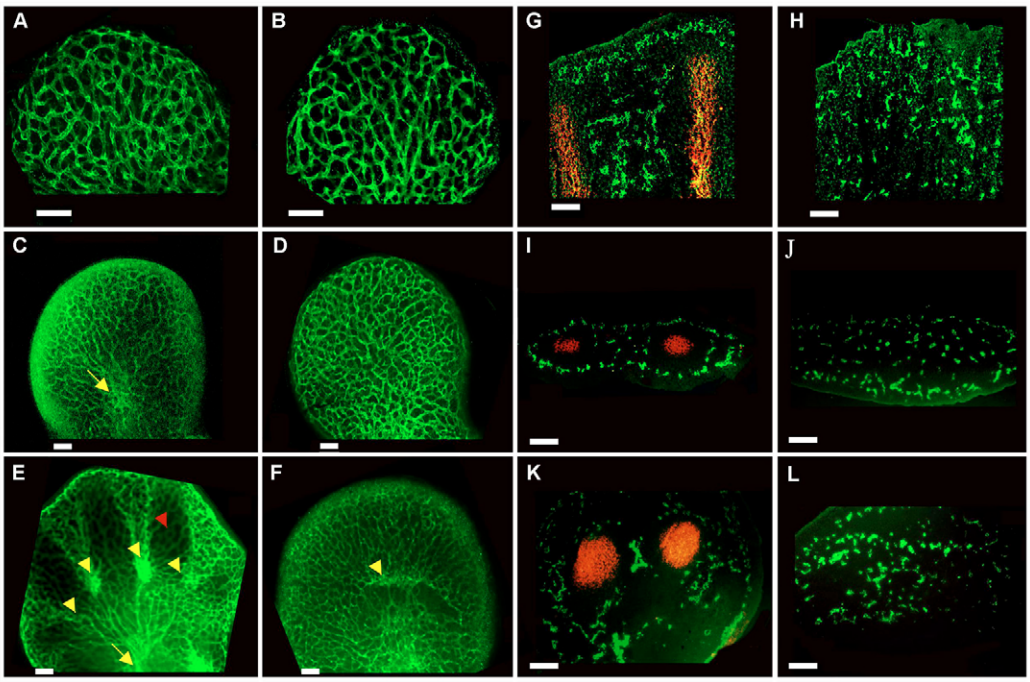


**Fig. 2. No vascular patterning in *Prx1-Sox9* limbs.** Knockout of *Sox9* expression in the limb mesenchyme before the formation of condensations prevented mesenchymal condensation and skeleton formation. Examination of E10.5-E12.5 control and *Prx1-Sox9* limb vasculature revealed that, in the absence of skeleton formation, the vasculature failed to pattern normally. (**A-F**) Whole limbs of control (A,C,E) and *Prx1-Sox9* (B,D,F) embryos immunostained with anti-CD31 antibody (green) for vascular endothelial cells. (A,B) E10.5; (C,D) E11.5; (E,F) E12.5. Yellow arrows indicate axial arteries; yellow arrowheads indicate metacarpal vascular centers in control limbs and a single center in *Prx1-Sox9* limbs; red arrowhead indicates avascular areas in control limbs. (**G-L**) Immunostaining of control (G,I,K) and *Prx1-Sox9* (H,J,L) sections with anti-CD31 for vascular endothelial cells (green) and anti-collagen II for chondrocytes (red). Longitudinal sections (G,H), and transverse sections of autopod (I,J) and zeugopod (K,L). Scale bars: 100 μm in A-F; 200 μm in G-L.

was observed at control limbs was missing from *Prx1-Sox9* limbs (Fig. 2C,D). At E12.5, instead of the highly branched metacarpal centers that were observed in control limbs, the *Prx1-Sox9* limb vessels formed a single wide center that spanned along the anterior-posterior axis of the limb (Fig. 2E,F). In addition, no avascular areas emerged as the *Prx1-Sox9* vasculature was evenly distributed throughout the autopod (Fig. 2H,J) and zeugopod (Fig. 2L), similar to the pre-condensation pattern we had observed in control limbs (Fig. 2A; Fig. 1A,E,H). These results demonstrate that the forming skeleton is necessary for limb vascular patterning, suggesting that the condensing mesenchyme produces a yet undescribed angiogenic signal that regulates this process.

## Spatially and temporally differential *Vegf* expression in condensing mesenchymal cells

The skeleton has been long known for its anti-angiogenic properties. Interestingly, the failure of the *Prx1-Sox9* limb vasculature to form a hierarchical pattern of a branched and dense vascular network strongly implies that the condensing mesenchymal cells also produce an angiogenic signal.

Previous works on mice that expressed only the heparin non-binding isoform of VEGF, namely VEGF120, identified abnormalities in the limb microvessel network, raising the hypothesis that VEGF, a key angiogenic factor, could be implicated in limb vascular patterning (Ruhrberg et al., 2002; Vieira et al., 2007). In order to test this

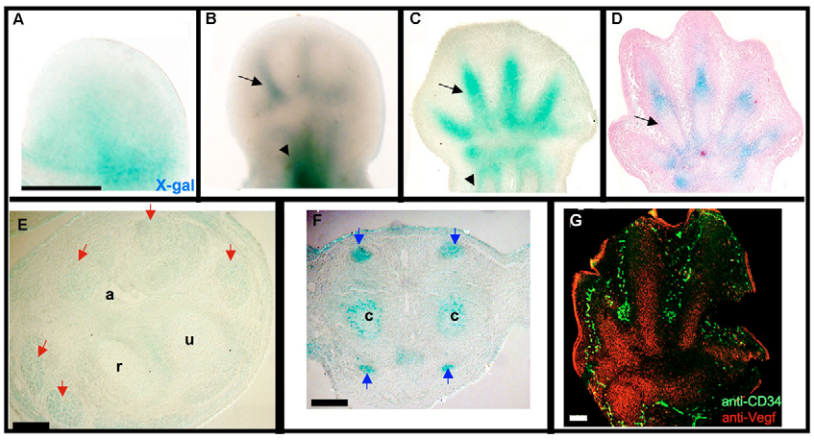


**Fig. 3. Expression of *Vegf* in condensed mesenchymal cells.** Using mice with an *IRES-LacZ* reporter cassette inserted into the 3′UTR of *Vegf* gene (*Vegf-lacZ*), we detected a dynamic expression of *Vegf* in the limb. (**A-D**) X-gal staining of E10.5 (A) and E11.5 (B) whole limbs, and E12.5 (C) and E13.5 (D) longitudinal sections. Black arrows indicate area of digit; black arrowheads indicate areas of radius and ulna. (**E**,**F**) X-gal staining of E13.5 transverse sections of the zeugopod, showing *Vegf-lacZ* expression in areas of forming muscles (red arrows, E), and of the autopod, showing *Vegf-lacZ* expression in areas of forming tendons (blue arrows, F; u, ulna; r, radius; a, artery; c, condensation). (**G**) Double immunofluorescence staining showing *Vegf* expression in areas several rows of cells away from endothelial cells, using anti-VEGF (red) and anti-CD34 (green) antibodies. Scale bars: 1 mm in A-D; 200 μm in E,F; 100 μm in G.

hypothesis, we analyzed *Vegf* expression in the developing limb, using mice with an IRES-*lacZ* reporter cassette inserted into the 3′UTR of the *Vegf* gene (*Vegf-lacZ*) (Miquerol et al., 1999). X-gal staining of whole limbs and sections revealed a dynamic expression of *Vegf* in the developing limb (Fig. 3A-F).

At E10.5, *Vegf* was expressed throughout the limb bud mesenchyme (Fig. 3A). At E11.5, *Vegf* expression was observed in the condensing mesenchyme of the radius and ulna, whereas in the condensing mesenchyme of the forming digits it was initiated (Fig. 3B). By E12.5, *Vegf* expression in the condensed mesenchymal cells of the digits was prominent. In the radius and ulna, where prechondrogenic cells have already differentiated to chondrocytes, *Vegf* expression was lost, whereas in the perichondrium around the forming cartilage it was maintained (Fig. 3C). At E13.5, *Vegf* expression was dramatically reduced in the digits, where the condensing cells have differentiated to chondrocytes, and was expressed only in the surrounding perichondrium and the forming joints, similar to its expression pattern in the zeugopod at E12.5 (Fig. 3D). In addition to its expression in the skeleton, we observed expression of *Vegf* in forming muscles and tendons (Fig. 3E,F). Interestingly, *Vegf* expression in the avascularized condensations formed domains that were located several rows of cells away from the flanking vasculature (Fig. 3G). Finding specific spatial and temporal *Vegf* expression in the condensing mesenchyme of the forming limb implicates *Vegf* as the proangiogenic signal by which the skeleton regulates limb vascular patterning.

## Overexpression of *Vegf* in condensed mesenchyme increases limb vascularization

To strengthen our hypothesis that the forming skeleton serves as a signaling center for the flanking vasculature by expressing *Vegf*, we used a gain-of-function approach. To examine the effect of overexpressing *Vegf* specifically in the forming condensation on limb vasculature, we used a triple transgenic system, in which the expression of the reverse tetracycline transactivator (*rtTA*) and the tetracycline-responsive element (*tetO-Vegf165*) transgene system was induced by *Sox9-Cre* (Akiyama et al., 2005; Belteki et al., 2005; Gossen et al., 1995) (for more details, see Materials and methods). qRT-PCR of E13.5 control and *Vegf*-overexpression forelimbs show a 1.7-fold increase in VEGF165 mRNA levels in the mutant, relative to the control. As the vasculature of the autopod is highly stereotypical, we concentrated on the effect of VEGF in that region.

The vasculature of *Vegf* overexpressed limbs was highly enriched, with endothelial cells that created denser and more complex networks in comparison with control limbs (Fig. 4A,B). Unlike in the control limbs, the metacarpal centers of *Vegf* overexpressed limbs were enriched with thicker vessels that originated in the axial artery (Fig. 4C,D). Moreover, these enriched metacarpal centers were wider and split into a denser and more complex network of small capillaries that occupied the interdigital areas (Fig. 4E,F). Interestingly, overexpression of *Vegf* in the condensing mesenchyme did not change the avascular properties of these areas, where no vessels were detected (Fig. 4G,H). These results support the hypothesis that VEGF produced in the avascularized condensations regulates the morphogenesis of the flanking vasculature.

## Lack of *Vegf* in limb mesenchyme results in absence of vascular morphogenesis

In order to examine directly the role of VEGF in limb vascular patterning, we took a loss-of-function approach. Having found that *Vegf* was expressed in the condensing mesenchyme as early

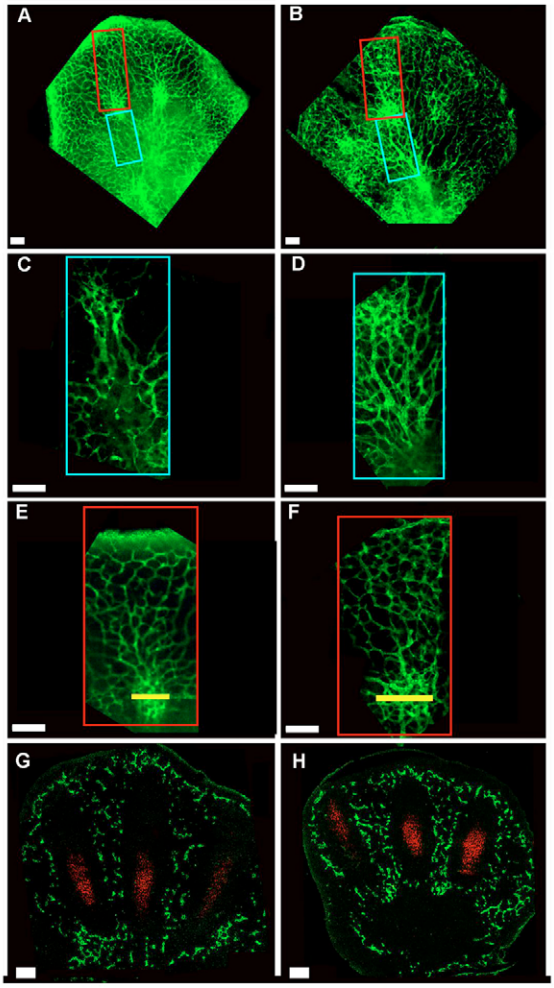


**Fig. 4. Overexpression of *Vegf* in condensed mesenchyme increases limb vascularization.** (**A**,**B**) Immunofluorescence staining with anti-CD31 (green), a marker for vascular endothelial cells, shows vasculature of E12.5 control (A) and *Vegf* overexpressed (B) whole limbs. (**C**,**D**) Enlargements of the blue boxes in A,B that demarcate metacarpal vascular centers; (**E**,**F**) enlargements of the red boxes in A,B that demarcate interdigital areas. (**G**,**H**) Immunostaining of control (G) and *Prx1-Sox9* (H) longitudinal sections with anti-CD31 for vascular endothelial cells (green) and anti-collagen II for chondrocytes (red). Scale bars: 100 μm.

as day 10.5, we used the *Prx1-Cre* mouse to delete *Vegf* in limb mesenchyme (Logan et al., 2002) (see Materials and methods). The reduction in *Vegf* expression was confirmed by qRT-PCR of E11.5 control and *Prx1-Vegf* limbs that demonstrated a 70% decrease in *Vegf* mRNA levels in *Prx1-Vegf* limbs, relative to the control. Vascular development and patterning was examined in E10.5-E12.5 whole limbs stained for endothelial cells using antibodies for CD31 (Fig. 5). In control limbs, we observed the stereotypical changes in vessel branching and complexity, namely the thickening of the axial artery and its split into thick vascular stems that supplied the interdigital zone (Fig. 5A,C,E). In *Prx1-Vegf* limbs, however, although the uniform capillary network and axial artery were formed, the capillary network branching was reduced, leading to a sparse network compared with control limbs (Fig. 5B,D,F). Longitudinal sections of *Prx1-Vegf* E12.5 limbs stained for endothelial cells demonstrated the absence of vascular

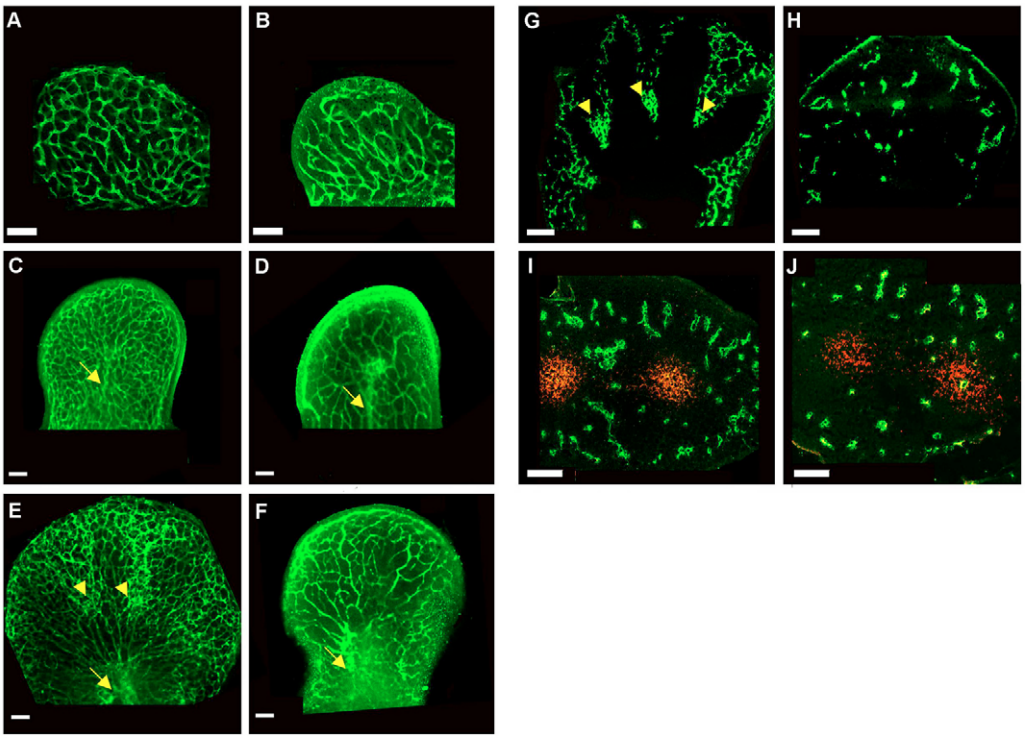


**Fig. 5. Lack of *Vegf* in limb mesenchyme results in an absence of vascular morphogenesis.** (**A-F**) Immunofluorescence with anti-CD31 for vascular endothelial cells (green) demonstrates the vasculature of control (A,C,E) and *Prx1-Vegf* (B,D,F) whole limbs at E10.5 (A,B), E11.5 (C,D) and E12.5 (E,F). (**G-J**) Longitudinal (G,H) and transverse (I,J) sections of E12.5 control (G,I) and *Prx1-Vegf* (H,J) limbs stained with anti-CD31 for vascular endothelial cells (G-J; green) and anti-collagen II for chondrocytes (I,J; red). Yellow arrows indicate axial arteries; yellow arrowheads indicate metacarpal vascular centers. Scale bars: 100 μm in A-F and I-J; 200 μm in G,H.

stems at the interdigital areas (Fig. 5G,H). Transverse sections stained for endothelial cells and chondrocytes showed a decrease in the number and diameter of vessels flanking the condensation areas of the forming digits (Fig. 5I,J). Similar to the control limbs, the vasculature regressed from the forming condensations in *Vegf*-ablated limbs. These results demonstrate that VEGF regulates vascular morphogenesis during limb vascular patterning.

## VEGF expressed in the avascular condensation affects limb vasculature via long-range interactions

Our results show that VEGF expressed in limb mesenchyme affects the vasculature located several rows of cells away from the *Vegf* source (Figs 4 and 5). Two distinct types of mechanisms could account for the ability of VEGF to regulate the remote vasculature. The first is a long-range mode of regulation, whereby VEGF formed in the condensation diffuses and affects the vasculature. Alternatively, there could be a relay mechanism that transfers the *Vegf* signal from the condensing mesenchyme to the vasculature. The latter would predict the expression of VEGF receptors in limb mesenchyme, thus enabling VEGF to induce the relay signal in the mesenchymal cells. We therefore examined the possibility that limb mesenchyme expresses the VEGF receptors *Flt1* (VEGFR1) and *Flk1* (VEGFR2), and the co-receptors neuropilin 1 (*Nrp1*) and neuropilin 2 (*Nrp2*). *Flt1*, *Flk1* and *Nrp1* expression was restricted solely to endothelial cells, whereas *Nrp2* had a broader expression pattern that also included mesenchymal cells (Fig. 6A).

As *Nrp1* and *Nrp2* are functionality redundant, in order to directly examine the possibility that *Nrp2* is involved in mediating *Vegf* signaling in mesenchymal cells, we blocked the expression of *Nrp1* in limb mesenchyme of *Nrp2*-null embryos (*Prx1-Nrp1*, *Nrp2*). Examination of limb vasculature of E13.5 *Prx1-Nrp1*, *Nrp2* embryos did not reveal any major abnormalities in vascular patterning, suggesting that in mesenchymal cells, *Nrp1* and *Nrp2* are not involved in the propagation of *Vegf* signaling to the limb vasculature (Fig. 6B). Although we failed to detect any expression of either *Flt1* or *Flk1* in limb mesenchyme, in order to exclude the possibility of sub-detectable, yet functionally significant, expression levels, we ablated *Flt1* and *Flk1* in limb mesenchyme. As expected, no major abnormalities were observed either in *Prx1-Flt1* or in *Prx1-Flk1* limbs (Fig. 6B). These results strongly imply that VEGF expressed by the condensation affects limb vasculature via long-range interactions.

## SOX9 is involved in the regulation of *Vegf* expression in condensing mesenchyme

The expression of *Vegf* by the condensing mesenchyme raised the hypothesis that SOX9 was involved in its regulation. This conjecture prompted us to examine the expression of the *Vegf-lacZ* reporter in *Prx1-Sox9* limbs (Fig. 7). *Vegf* expression was indeed reduced, most prominently at E12.5, when it could only be observed in a few cells located in the center of the limb (Fig. 7A,B). To further validate the possibility that SOX9 regulates *Vegf* in the condensing mesenchyme, we used a high-density micromass culture as an in vitro model (DeLise et al., 2000). Micromass cultures derived from limb buds of *floxed-Sox9* embryos were infected by either adeno-Cre virus (AdCre) to delete *Sox9*, or with β-Gal-expressing adenovirus (AdβGal) as a control. To assess the efficiency of *Sox9* deletion by AdCre, we used quantitative real-time PCR. The expression level of *Sox9* in AdCre-infected cells was reduced by 86% relative to the control cells, suggesting an efficient blockage of *Sox9* (Fig. 7C). Next, we examined the expression of *Vegf* transcript, which was reduced by 70% compared with control cells (Fig. 7D).

Finally, to determine whether or not SOX9 is sufficient to regulate the expression of *Vegf* in limb mesenchyme, we used transgenic mice in which *Sox9* expression is under the control of the *Prx1* regulatory sequence (Akiyama et al., 2005), thereby ectopically expressed in limb mesenchyme. Next, we examined the expression of *Vegf* in sections of E12.5 *Sox9*-misexpressing and control

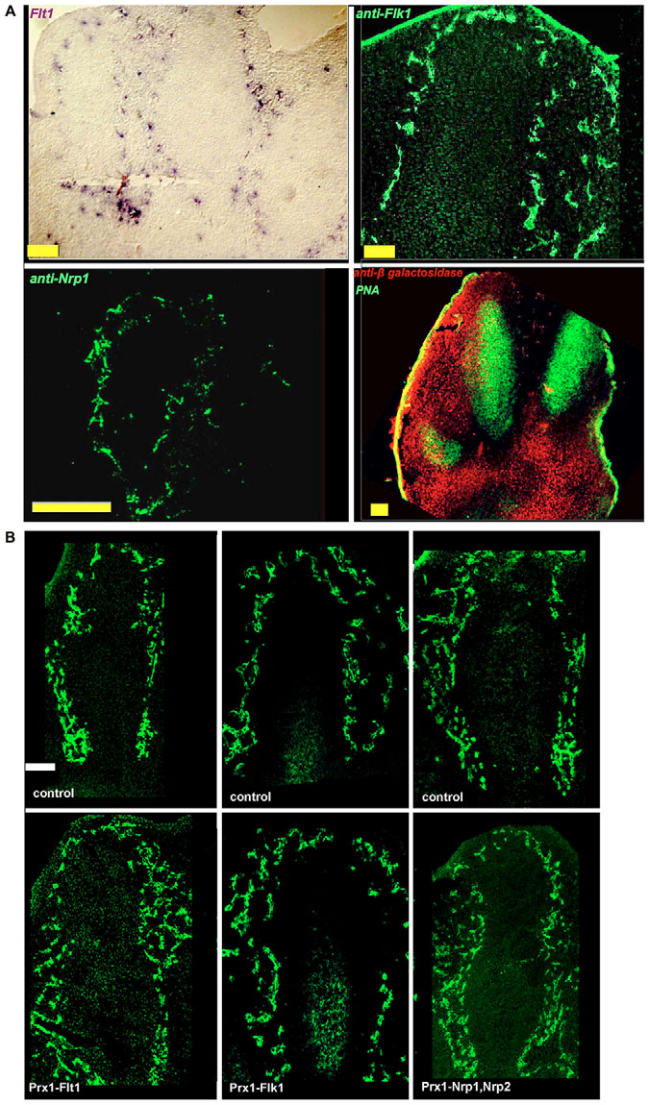


**Fig. 6. Expression patterns of VEGF receptors in the limb.** (**A**) Detection of VEGF receptors in E12.5 longitudinal sections: in situ hybridization of *Flt1* and immunofluorescence of *Flk1* using RAFL-1 antibody and of *Nrp1* using anti-NRP1 antibody show their expression in endothelial cells. Immunofluorescence of *Nrp2* (red) and condensed mesenchyme (green) using anti-β-galactosidase and biotin labeled peanut agglutinin, respectively. Scale bars: 100 μm in *Flt1*, *Flk1, Nrp2*; 50 μm in *Nrp1*. (**B**) Immunofluorescence staining with anti-CD31 (green) as a marker for vascular endothelial cells reveals that an ablation of VEGF receptors in mesenchyme does not affect vascular patterning: E12.5 longitudinal sections of control and *Prx1-Flt1*, *Prx1-Flk1* and *Prx1-Nrp1, Nrp2* limbs. Scale bar: 100 μm.

forelimbs using immunofluorescence staining with anti-VEGF antibody. Our results show that *Vegf* expression was maintained in the condensation areas of the future digits, similar to its expression pattern in control limbs. No staining was observed in areas of the limb that exhibited ectopic *Sox9* misexpression (Fig. 7E). In addition, we examined vascular patterning in E12.5 *Sox9*-misexpressing forelimbs by whole-mount immunofluorescence staining with anti-CD31 antibody. No major changes were detected in vascular branching and morphogenesis of *Sox9*-misexpressing limbs (Fig. 7F). These experiments emphasize SOX9 involvement in *Vegf* expression; however, they also show that SOX9 is not sufficient to induce *Vegf* expression in limb mesenchyme, suggesting its dependence on other factor or factors that are localized to the condensation.

## DISCUSSION

In this study, we demonstrate a novel role for the limb skeleton as a signaling center for vascular patterning by expressing *Vegf*. In the absence of skeleton formation, limb vasculature failed to pattern normally. Moreover, both *Vegf* loss- and gain-of-function in the forming limb skeleton affected vessel morphogenesis. The normally patterned vasculature observed upon inactivation of VEGF receptors *Flt1*, *Flk1*, *Nrp1* and *Nrp2* in limb mesenchyme suggests that VEGF regulates limb vasculature via a long-range mechanism. Finally, finding that SOX9 is involved in the regulation of *Vegf* in condensing mesenchymal cells provides a mechanism that coordinates the genetic programs of skeleton development and vascular patterning by a shared transcriptional regulation.

### The skeleton serves as a signaling center for the patterning of limb vasculature

The coordination among bones, muscles, tendons, nerves and blood vessels in the developing limb is needed for normal development and functionality. Although much has been learned in recent years about the signals that orchestrate limb patterning and morphogenesis, the mechanism that underlies the specific patterning of limb vasculature and its coordination with other tissues that compose the limb has remained poorly understood.

Several models can account for coordinated patterning of the skeleton and its vasculature. First, the developing skeleton may regulate vascular patterning; second, the developing vasculature may regulate skeletal patterning; and, third, both tissues may respond to common signals that originate in other tissues. This study provides direct evidence for the centrality of the skeleton in limb vasculature patterning. Once skeletogenesis is initiated, the limb vasculature undergoes an extensive rearrangement that involves two seemingly opposing processes. The first process is regression of vessels from the sites of condensation, which renders them avascular. Concurrently, the surrounding vasculature undergoes extensive morphogenesis to form a stereotypical, highly complex branched network (Fig. 1). However, in the absence of mesenchymal condensation and skeleton formation, both vessel regression and morphogenesis were lost (Fig. 2). These findings strongly support the active regulatory role of the limb skeleton in patterning limb vasculature. Moreover, these results imply the existence of a previously unappreciated signal from the forming skeleton to the endothelial cells of the limb vasculature. Hence, the forming skeleton serves as a signaling center that regulates limb vasculature.

Further support for the centrality of the skeleton as a regulator of limb vasculature comes from studies on the involvement of the musculature in limb vasculature. Although the musculature is, like the skeleton, a central component of the limb, its absence does not have dramatic effect on limb vascular development (De Angelis et al., 1999).

The evolutionary driving force that selected the skeleton as a signaling center for limb vasculature is unclear. One plausible explanation for this selection lies in the mechanism of skeleton formation. As shown in this work and unlike numerous other tissues, mesenchymal condensations develop in an avascular environment (Fig. 1F,G). Thus, the formation of the skeleton dictates its segregation from the vasculature by regression of vessels from condensation areas. Yet, to ensure sufficient supply of nutrients and

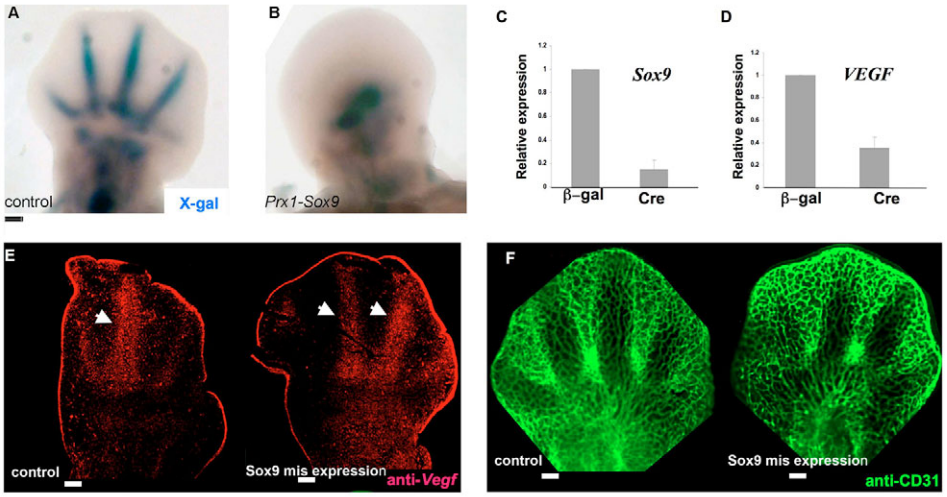


**Fig. 7. SOX9 is involved in *Vegf* expression in limb mesenchyme.** (**A**,**B**) Detection of *Vegf* expression in E12.5 control (A) and *Prx1*-Sox9 whole limbs (B) using X-gal staining. (**C**,**D**) qRT-PCR on AdβGal (control) and AdCre infected micromass cultures show relative expression levels of *Sox9* (C) and *Vegf* (D) mRNA transcripts. (**E**) Immunofluorescence staining of *Vegf* in the autopod of E12.5 control and *Sox9*-misexpressing limbs, using anti-VEGF antibody. White arrowheads indicate areas of *Vegf* expression in the digits. (**F**) Vascular patterning of E12.5 control and *Sox9*-misexpressing limbs as visualized by whole-mount immunofluorescence staining with anti-CD31 antibody. Scale bars: 100 μm.

oxygen while it is segregated from the vasculature, the skeleton has adopted a mechanism that compensates for vessel regression by inducing vascular morphogenesis in its vicinity.

Nonetheless, another evolutionary question that remains unanswered is why mesenchymal condensation requires avascular hypoxic conditions. An indication for the evolutionary motivation may be offered by our previous study, in which we demonstrated that hypoxia, generated by the segregation of the vasculature from the skeleton, regulated the differentiation of condensing mesenchymal cells to chondrocytes. As a molecular mechanism, we demonstrated that the transcription factor complex hypoxia-inducible factor 1 (HIF1), a key mediator of adaptive responses to changes in cellular oxygen levels (Semenza, 1998), regulated the expression of *Sox9* in hypoxic prechondrogenic cells (Amarilio et al., 2007; Provot et al., 2007).

Although our results clearly demonstrate that the skeleton regulates vascular patterning, we cannot exclude the possibility that the vasculature has a reciprocal role in regulating skeletogenesis; in fact, we favor this hypothesis. Previous studies demonstrating the important role of the vasculature in skeleton development support this hypothesis (Yin and Pacifici, 2001; Fraser and Travill, 1978; Feinberg and Saunders, 1982; Hootnick et al., 1980). The advantage of crossregulation between the forming skeleton and its vasculature is higher levels of flexibility and subtle coordination between the tissues, which are required for proper limb development and functionality.

## VEGF expressed in condensing mesenchyme regulates limb vascular morphogenesis

The prevailing model for limb vascularization is based on three elements: the inherent tendency of the endothelium to continuously divide and branch, resulting in the formation of a dense vascular network; a shared response of the limb endothelium and the mesenchymal cells to mitotic stimuli; and the anti-angiogenic properties of the forming skeleton, which are responsible for the formation of avascular regions (Belteki et al., 2005; Caplan, 1985). The predicted outcome of this model would be either poor vascularization in the vicinity of the condensation, assuming that the anti-angiogenic signal is mediated by a soluble molecule, or an even distribution of vessels outside the avascular condensation, if the signal is strictly localized to the forming skeleton. One obvious problem with such a model is the lack of a compensating mechanism that ensures sufficient supply of nutrients and oxygen to the segregated avascular condensation. Now, our results challenge this model, specifically the claim that vascular morphogenesis is solely the result of the intrinsic property of endothelial cells. Instead, we argue that, in addition to its anti-angiogenic signal, the condensing mesenchyme in the forming limb produces a yet undescribed pro-angiogenic signal.

The expression of *Vegf*, a key angiogenic regulator, in the condensing mesenchyme suggests the involvement of VEGF in limb vasculature patterning. Previous examples of VEGF involvement in vascular patterning have been provided by studies on avian embryos, in which loss and gain of function of *Vegf* resulted in severe alteration in the patterning of the vascular plexus (Drake, 1995; Drake, 2000). The alterations in limb vasculature we witnessed in both loss- and gain-of-function experiments on *Vegf* in limb mesenchyme strongly supported this hypothesis (Figs 4 and 5). Interestingly, the expression of *Vegf* in the condensing mesenchyme was temporal: once the vasculature has become patterned and the mesenchymal cells have differentiated to chondrocytes, *Vegf* expression is reduced and can only be observed in the forming perichondrium and joints (Fig. 3C,D). The expression of *Vegf* in differentiated chondrocytes will be elevated again later in development, in order to ensure the invasion of blood vessels during endochondral bone formation (Zelzer et al., 2004; Zelzer et al., 2002).

The mechanism that regulates the spatial and temporal expression of *Vegf* in the condensing mesenchyme remains unidentified. Although it is clear that SOX9 plays a role in *Vegf* expression, the fact that *Sox9* expression, in contrast to *Vegf* expression, is maintained in differentiated chondrocytes clearly indicates the involvement of additional transcriptional components in *Vegf* regulation. The ability of SOX9 to drive the expression of *Vegf* only in the condensing mesenchyme (Fig. 7) further supports this notion. Nevertheless, our finding that SOX9, an essential factor of mesenchymal condensation, is involved in the regulation of *Vegf* expression implies that the genetic program that controls the initial stage in skeletogenesis also regulates vascular development, thus ensuring a tight coordination in the development of both systems.

During organogenesis, the adaptation of the vasculature to the growing demands of the developing organ for oxygen and nutrient supply is crucial. Organs such as lung, liver, kidney and pancreas develop with an embedded vasculature (Cleaver and Melton, 2003; Coultas et al., 2005), suggesting that the mechanism that synchronizes their development with the vasculature is based on intimate cellular interaction. *Vegf* expression in the avascularized

condensing mesenchyme represents a different mode of vascular regulation by VEGF. Based on the well-established ability of VEGF to induce the formation and recruitment of blood vessels (Carmeliet, 2005; Ferrara, 2004), it was expected that sites with the highest level of *Vegf* expression would be mostly enriched with blood vessels. The expression of *Vegf* in condensed mesenchyme, several rows of cells away from the flanking vasculature, implies that VEGF-mediated regulation of vascular morphogenesis may operate either via a relay mechanism, or as a direct long-range mechanism. The apparently patterned vasculature in limbs where the expression of VEGF receptors was blocked in mesenchyme (Fig. 6B) rules out the possibility of a relay mechanism and favors a long-range direct regulation of VEGF on limb vasculature.

Long-range regulation of vascular patterning by VEGF has previously been demonstrated in the formation of the perineural vascular plexus (PNVP) that encompasses the neural tube. *Vegf* expression in the neural tube induced the migration and assembly of presomitic mesoderm angioblasts of the PNVP (Hogan et al., 2004). Another example for a long-range regulation by VEGF was given by experiments that used VEGF-coated beads, in which vessel formation was also observed in the vicinity of the planted beads (Bates et al., 2003; Finkelstein and Poole, 2003). A possible mechanistic explanation for the long-range effect of VEGF is based on the formation of several isoforms of VEGF, which exhibit different diffusion properties (VEGF120, VEGF164 and VEGF188) (Ferrara and Davis-Smyth, 1997; Ferrara et al., 1992; Park et al., 1993; Shima et al., 1996). Vascular abnormalities in limbs of mice expressing only the VEGF120 isoform (Ruhrberg et al., 2002; Vieira et al., 2007), as well as our observation that all three isoforms of VEGF are expressed in E12.5 limbs (data not shown) raise the hypothesis that some aspects of VEGF regulation of limb vasculature are mediated by the different isoforms.

The mechanism of vessel regression and segregation from mesenchymal condensations remains largely unknown. Our finding that this process proceeded normally in limbs where *Vegf* was either depleted (Fig. 5) or overexpressed (Fig. 4) indicates that VEGF has no role in the mechanism that underlies this phenomenon. Moreover, these experiments indicate that the process of vessel regression and segregation is not coupled with vessel morphogenesis.

During organogenesis, it is cardinal that the vasculature accommodates the growing metabolic needs of the developing organ. Interestingly and contra-intuitively, several organs, including the skeleton, develop in the absence of embedded vasculature. The segregation between the forming skeleton and its vasculature requires the existence of a genetic program that would synchronize organ development with its non-embedded vasculature. In this manuscript, we suggest a paradigm for a mechanism that allows for the coordination of skeleton development and vascular patterning by establishing the skeleton as a signaling center that regulates limb vasculature.

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