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# FGF18 is required for early chondrocyte proliferation, hypertrophy and vascular invasion of the growth plate

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**Abstract**

Fibroblast growth factor 18 (FGF18) has been shown to regulate chondrocyte proliferation and differentiation by signaling through FGF receptor 3 (FGFR3) and to regulate osteogenesis by signaling through other FGFRs. *Fgf18*$^{-/-}$ mice have an apparent delay in skeletal mineralization that is not seen in *Fgfr3*$^{-/-}$ mice. However, this delay in mineralization could not be simply explained by FGF18 signaling to osteoblasts. Here we show that delayed mineralization in *Fgf18*$^{-/-}$ mice was closely associated with delayed initiation of chondrocyte hypertrophy, decreased proliferation at early stages of chondrogenesis, delayed skeletal vascularization and delayed osteoclast and osteoblast recruitment to the growth plate. We further show that FGF18 is necessary for *Vegf* expression in hypertrophic chondrocytes and the perichondrium and is sufficient to induce *Vegf* expression in skeletal explants. These findings support a model in which FGF18 regulates skeletal vascularization and subsequent recruitment of osteoblasts/osteoclasts through regulation of early stages of chondrogenesis and VEGF expression. FGF18 thus coordinates neovascularization of the growth plate with chondrocyte and osteoblast growth and differentiation.


*Keywords:* Fibroblast growth factor 18 (FGF18); Skeletal development; Growth plate; Chondrocyte; Osteoblast; Periosteum; Perichondrium; Vascular development

## Introduction

Skeletogenesis in vertebrates is achieved through two distinct developmental processes. Cranial bones and medial clavicles are formed through intramembranous ossification, while facial bones and the appendicular skeleton develop through endochondral ossification (Erlebacher et al., 1995; Karsenty and Wagner, 2002). Shortly after limb bud formation, endochondral ossification is initiated from mesenchymal condensations. Differentiation of condensed mesenchyme gives rise to chondrocytes centrally and osteoprogenitors in the perichondrium. Proliferating chondrocytes and subsequently differentiated hypertrophic chondrocytes elaborate an extracellular matrix (ECM) that forms a cartilaginous template for future mineralization.

Fibroblast growth factors (FGFs) are signaling molecules that are essential regulators of endochondral bone growth. FGF receptor 3 (FGFR3) is expressed in proliferating chondrocytes where it regulates proliferation and differentiation. At late embryonic stages and during postnatal skeletal growth, FGFR3 signaling suppresses chondrocyte proliferation and hypertrophic differentiation (Goldring et al., 2006; Ornitz and Marie, 2002). However, during early skeletal development, FGFR3 is mitogenic for chondrocytes and inhibits hypertrophic differentiation (Iwata et al., 2000). FGF18 is expressed in the perichondrium and in joint spaces and has been identified as a potential ligand for FGFR3 (Davidson et al., 2005; Xu et al., 2000). At late embryonic stages, both *Fgf18*$^{-/-}$ and *Fgfr3*$^{-/-}$ mice have an expanded proliferating and hypertrophic chondrocyte zone, associated with increased Indian hedgehog (IHH) expression and signaling (Colvin et al., 1996; Liu et al., 2002; Naski et al., 1998; Ohbayashi et al., 2002). These similarities suggest that FGF18 is a functional ligand for FGFR3. However, *Fgf18*$^{-/-}$ mice have reduced bone length and show an approximate 2-day delay in the onset of skeletal mineralization, phenotypes that are not observed in *Fgfr3*$^{-/-}$ mice (Liu et al.,

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2002). This suggests that FGF18 signals to other FGFRs to regulate the onset of the mineralization process. The delayed mineralization phenotype in *Fgf18*$^{-/-}$ mice could be explained by either a direct effect of FGF18 on the chondrocyte and osteoblast or an indirect effect of FGF18 on, for example, vascularization of the growth plate.

The hypertrophic chondrocyte zone is an avascular structure that must be invaded by blood vessels that arise from the perichondrium. This neovasculogenesis of the central hypertrophic zone allows hematopoietic-derived osteoclasts/chondroclasts to enter and progressively erode the cartilaginous matrix (Goldring et al., 2006; Kronenberg, 2003; Wagner and Karsenty, 2001; Zelzer and Olsen, 2005). Along with the vasculature, perichondrial-derived osteoblasts eventually replace the eroded cartilaginous matrix with trabecular bone. Such a sequence of events relies on the precise coupling of chondrogenesis, osteogenesis and vascularization. However, it is still unclear how these different developmental processes are coordinated in a temporally and spatially correct fashion.

The endothelial cell-specific mitogen vascular endothelial growth factor (VEGFA) is an important regulator of vasculogenesis and angiogenesis during endochondral ossification. VEGFA binds to its tyrosine kinase receptors VEGFR1 (Flt1, VR1) and VEGFR2 (Flk1, VR2) (Yancopoulos et al., 2000). Administration of a soluble receptor to inhibit VEGF suppresses vascular invasion and results in bone length reduction and hypertrophic zone expansion (Gerber et al., 1999). Conditional targeting of VEGFA or VEGF164/188 in type II collagen-expressing cells and descendants produces similar phenotypes (Maes et al., 2002; Zelzer et al., 2002). These data demonstrate that VEGF/VEGFR signaling is essential for skeletal vascularization.

In this study, we demonstrate that the delayed ossification observed in *Fgf18*$^{-/-}$ mice was closely associated with delayed initiation of chondrocyte hypertrophy, skeletal vascularization and osteoclast recruitment, and decreased chondrocyte proliferation. We show that expression of *Vegf* in hypertrophic chondrocytes was also delayed in *Fgf18*$^{-/-}$ mice with concurrent decreased expression of *Vegfr1*. In an *in vitro* limb explant culture system, we demonstrated that FGF18 was sufficient to induce *Vegf* expression. These findings support a model in which FGF18 regulates skeletal vascularization and osteoclast recruitment through regulation of VEGF signaling. Combined with previous data, we conclude that FGF18 is an essential regulator of skeletogenesis by coordinating chondrocyte proliferation/differentiation, osteoblast proliferation and vascular invasion.

## Materials and methods

### *Skeletal preparations*

Skeletons were prepared as described previously (Liu et al., 2002). For postnatal day 0 (P0) skeletal preparations, carcasses were skinned and eviscerated, and then soaked in acetone for 12–24 h, cleared in 2% KOH (12–24 h), stained with alizarin red S and alcian blue (12–24 h), cleared in 1% KOH/20% glycerol, and stored in glycerol. For embryo skeleton preparations, fetuses were skinned and eviscerated, stained with alizarin red S and alcian blue (12–24 h), cleared in 1% KOH/20% glycerol, and stored in glycerol.

### *Histological and immunohistochemical analysis*

Tissues were fixed in 4% paraformaldehyde/PBS, decalcified if necessary in EDTA or Decalcifying Solution (Stephens Scientific), and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E), von Kossa and TRAP. For PECAM (CD31) immunohistochemistry, sections were deparaffinized with two changes of xylene for 10 min each, then rehydrated through 100%, 95%, 75% and 50% ethanol washes for 5 min each. After rinsing in PBS, sections were treated with 0.1% trypsin at 37 °C for 10 min and rinsed in three changes of PBS for 5 min each. Sections were blocked in 5% serum for 30 min and then incubated with anti-PECAM antibody (BD Pharmingen) at 4 °C for 12–24 h, rinsed with PBS, and incubated with secondary antibody (Chemicon) at RT for 1 h. Fluorescent microscopic images were taken using a Hamumatsu camera and AxioVison3.0 software (Zeiss).

### *In situ hybridization*

*In situ* hybridization was performed as described previously (Liu et al., 2002). The plasmids used for generating $P^{33}$-labeled riboprobes were generously provided by: B. Olsen (*Collagen X*, *Vegfr1*, *Vegfr2*); G. Karsenty (*Vegf*); G. Andersson (*Trap*); Z. Werb (*Mmp9*); A. McMahon (*Ihh*); H. Kronenberg (*Pth1r*); B. de Crombrugghe (*Sox9*, *Collagen 1*).

### *Real-time quantitative PCR*

Total RNA was extracted from E16.5 skinned autopods using the RNeasy Mini kit (Qiagen, Valencia, CA). Two RNA samples from each genotype were pooled together, 1 μg of total RNA was used for oligo dT or random hexanucleotide-primed cDNA synthesis. cDNAs were added to 25 μl reaction mixtures containing 12.5 μl of SYBR Green master mix (Applied Biosystems, Foster City, CA), 0.25 U of UDP-N-glycosidase (Invitrogen), and 400 nM gene-specific primers. A melting curve was used to identify a temperature where only the amplicon, and not primer dimers, accounted for the SYBR green-bound fluorescence. Assays were performed in quadruplicate with an ABI Prism 7700 Sequence Detector (Applied Biosystems). All data were normalized to an internal standard (GAPDH; comparative $C_T$ method, User Bulletin 2, Applied Biosystems).

Primer sequences:*Gapdh* (5′ TGCACCACCAACTGCTTAG 3′; 5′ GGATGCAGGGATGATGTTC 3′), *Flt1* (5′ GAGGAGGATGAGGGTGTCTATAGGT 3′; 5′ GTGATCAGCTCCAGGTTTGACTT 3′) (Shih et al., 2002), *Flk1* (5′ AGGTCACCATTCATCGCCTC 3′; 5′ GAAATCGACCCTCGGCATG 3′), *Pecam1* (5′ AGGAAAGCCAAGGCCAAACA 3′; 5′ CATTAAGGGAGCCTTCCGTTCT 3′) (Fitzsimmons et al., 2000), *VegfA* (5′ GGAGATCCTTCGAGGAGCACTT 3′; 5′ GGCGATTTAGCAGCAGATATAAGAA 3′) (Shih et al., 2002), *VegfB* (5′ GAAGAAAGTGGTGCCATGGATAG 3′; 5′ CCCATGAGTTCCATGCTCAGA 3′) (Mills et al., 2001).

PCR conditions for *Flt1* and *Flk1*: 50 °C 2 min; 95 °C 10 min; 35 (95 °C 15 s; 60 °C 1 min). PCR conditions for *VegfA, VegfB*: 95 °C 3 min; 40 (95 °C 30 s; 60 °C 30 s; 72 °C 30 s).

### *Embryonic limb explant cultures and whole mount β-galactosidase staining*

Limb explant cultures were carried out essentially as described (Minina et al., 2001) with minor modifications. Forelimb cartilage was dissected from E14.5 VEGF-*LacZ* or wild type mouse embryos (Miquerol et al., 1999) and placed at the air-fluid interface on Transwell filters (Corning) containing DMEM, 10% fetal calf serum (GibcoBRL), antibiotic/antimycotic solution (Sigma) and 2 μg/ml heparin. Media was supplemented with 250 ng/ml recombinant FGF10 (Peprotech), recombinant FGF18 (Peprotech), or BSA. Explants were cultured for 24 h or 48 h at 37°C/5% $CO_2$ under humidified conditions. For whole mount β-galactosidase staining, limb explants were harvested on Day 2 and fixed in 0.2% glutaraldehyde, 5 mM EGTA pH 7.3, 100 mM $MgCl_2$, 0.02% NP40, 0.01% sodium deoxycholate in PBS. After washing with PBS, explants were incubated for 4–5 h at 24 °C in β-galactosidase staining buffer containing 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$,