

RESEARCH REPORT

Vegfa regulates perichondrial vascularity and osteoblast differentiation in bone development

Xuchen Duan, Yurie Murata, Yanqiu Liu, Claudia Nicolae, Bjorn R. Olsen and Agnes D. Berendsen*

ABSTRACT

Vascular endothelial growth factor A (Vegfa) has important roles in endochondral bone formation. Osteoblast precursors, endothelial cells and osteoclasts migrate from perichondrium into primary ossification centers of cartilage templates of future bones in response to Vegfa secreted by (pre)hypertrophic chondrocytes. Perichondrial osteolineage cells also produce Vegfa, but its function is not well understood. By deleting *Vegfa* in osteolineage cells *in vivo*, we demonstrate that progenitor-derived Vegfa is required for blood vessel recruitment in perichondrium and the differentiation of osteoblast precursors in mice. Conditional deletion of Vegfa receptors indicates that Vegfa-dependent effects on osteoblast differentiation are mediated by Vegf receptor 2 (Vegfr2). In addition, Vegfa/Vegfr2 signaling stimulates the expression and activity of Indian hedgehog, increases the expression of β -catenin and inhibits Notch2. Our findings identify Vegfa as a regulator of perichondrial vascularity and osteoblast differentiation at early stages of bone development.

KEY WORDS: Vascular endothelial growth factor A, Osteoblast differentiation, Bone development, Osteoblast precursor, Osterix, Indian hedgehog, β -catenin, Notch, Mouse

INTRODUCTION

During endochondral bone formation osteochondroprogenitors form cartilage templates of future bones. Following chondrocyte hypertrophy, perichondrial osteoblast precursors, endothelial and hematopoietic cells and osteoclasts migrate into primary ossification centers (POCs) and replace cartilage by bone marrow and trabecular bone (Karsenty, 2003; Kronenberg, 2003; Zelzer and Olsen, 2003).

The transcription factor osterix (Osx; Sp7 – Mouse Genome Informatics) is expressed in perichondrial osteoblast precursors and, at lower levels, in prehypertrophic chondrocytes (Nakashima et al., 2002). Osx expression first appears in the perichondrium of bone templates at embryonic day (E) 13.5 in mice. After E15.5, strong expression is associated with trabecular and cortical bone formation (Nakashima et al., 2002) as the precursors differentiate into collagen I (Col1)-expressing osteoblasts (Karsenty and Wagner, 2002; Maes et al., 2010b).

Several factors regulate endochondral bone formation, including Indian hedgehog (Ihh), Wnt/ β -catenin, Notch, Bmps, Fgfs and Pthrp (Kronenberg, 2003). Ihh induces osteoblast differentiation during perichondrial maturation into periosteum (Chung et al., 2001; Hu et al., 2005; Long et al., 2004). Canonical Wnt/ β -catenin signaling is essential for the differentiation of osteochondroprogenitors (Day

et al., 2005; Hill et al., 2005) and promotes hypertrophic chondrocyte differentiation and osteoblast differentiation and maturation (Hu et al., 2005). Notch2 is a negative regulator of osteoblast differentiation (Hilton et al., 2008).

Vegfa is essential during key stages of endochondral bone formation. Mice with *Vegfa* conditionally deleted in collagen II (Col2)-expressing cells show a delay in blood vessel invasion into POCs, delayed hypertrophic cartilage removal and chondrocyte apoptosis (Haigh et al., 2000; Zelzer et al., 2002, 2004). Mice expressing only the non-heparin-binding Vegf120 isoform of Vegfa show decreased skeletal mineralization and reduced expression of osteoblastic markers (Maes et al., 2002; Zelzer et al., 2002). Overexpression of Vegfa in osteochondroprogenitor cells results in increased bone mass (Maes et al., 2010a).

Osx-positive osteoblast precursors not only migrate into POCs in response to Vegfa secreted by chondrocytes, but they also express high levels of Vegfa (Maes et al., 2010b). In this study, we show that Vegfa produced by these cells is required for blood vessel recruitment and early stages of osteoblast differentiation in perichondrial regions of bone templates. This function is mediated by Vegfr2 and is likely to involve Ihh, β -catenin and Notch2-dependent pathways.

RESULTS AND DISCUSSION

Vegfa regulates bone formation during endochondral ossification

To assess the function of Vegfa expressed by Osx-positive precursor cells in developing bone, we generated mice with conditional loss of *Vegfa* alleles in these cells. Newborn *Vegfa^{fl/fl}; Osx-Cre:GFP* mice had thinner bones and reduced skeletal mineralization compared with wild-type (WT) (*Vegfa^{+/+}; Osx-Cre:GFP*) mice (Fig. 1A,B). MicroCT showed reduced tibia length and that secondary ossification centers were largely missing in mutants at postnatal day (P) 9 (supplementary material Fig. S1A). Mutant femurs had increased hypertrophic zones, decreased mineralization and fewer tartrate-resistant acid phosphatase (Trap)-positive osteoclasts and GFP-labeled Osx-expressing (*Osx/GFP⁺*) cells (Fig. 1B). Anti-CD31 (Pecam1) staining indicated reduced density of endothelial and *Osx/GFP⁺* cells within primary spongiosa in mutants (Fig. 1B).

Visualization of *Vegfa* expression by X-gal staining of P1 femurs from heterozygous *Vegfa-lacZ^{KI/WT}* (*Vegfa-lacZ*) mice revealed positive staining of prehypertrophic chondrocytes and cells located within primary spongiosa; by contrast, WT tissue showed only low-level false-positive staining in osteoclasts (supplementary material Fig. S1B). *In situ* hybridization indicated that loss of Vegfa expression in Osx⁺ precursors resulted in reduced *Col1* (*Colla1*)-expressing cells within primary spongiosa (Fig. 1C). Low *Coll* expression in mutant bones confirmed that the majority of *Osx/GFP⁺* cells are osteoblast progenitor cells, which are decreased in mutants (Fig. 1B,C).

Department of Developmental Biology, Harvard School of Dental Medicine, Boston, MA 02115, USA.

*Author for correspondence (Agnes_Berendsen@hsdm.harvard.edu)

Received 17 September 2014; Accepted 19 April 2015

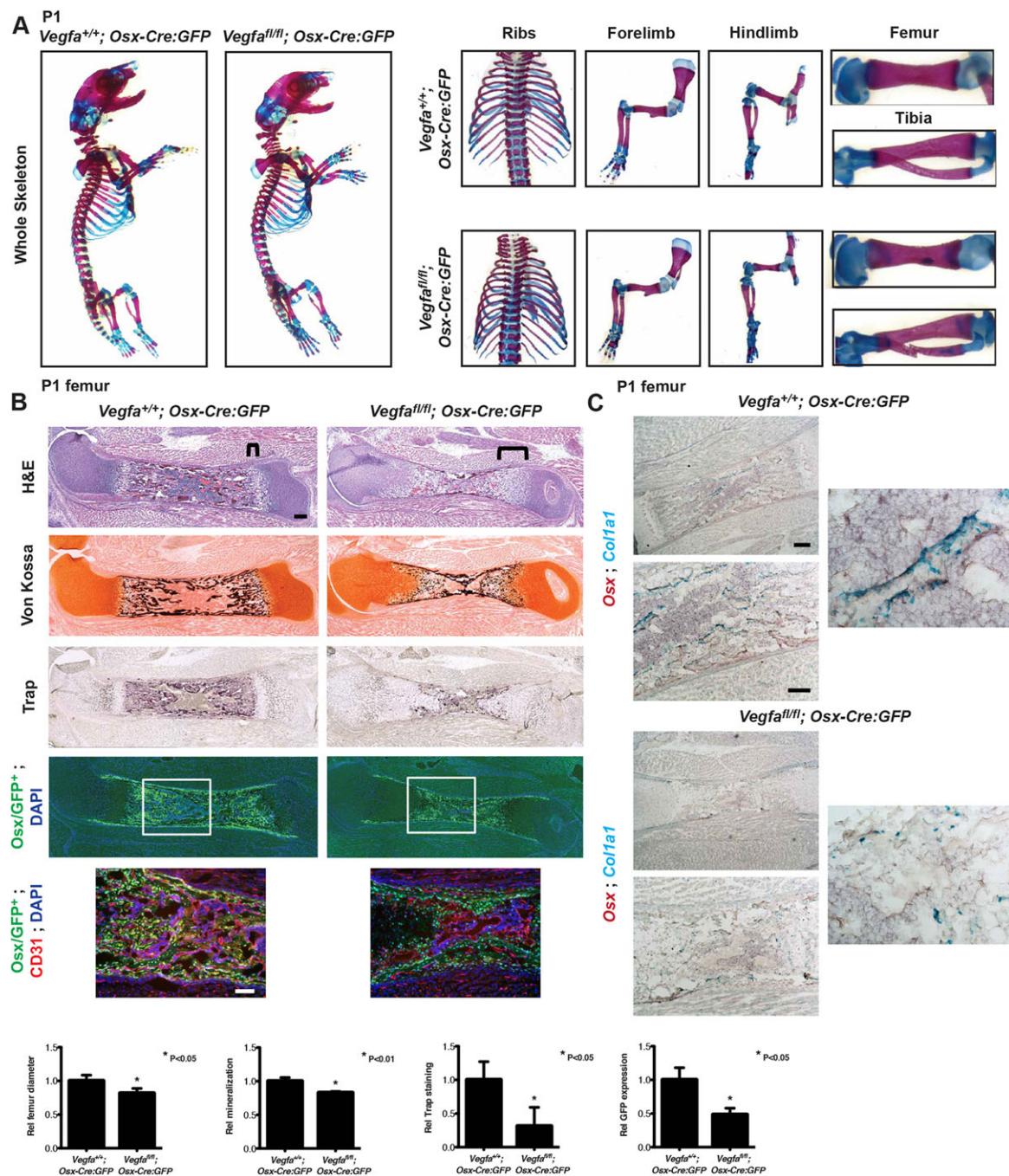


Fig. 1. Progenitor-derived Vegfa regulates mineralization in developing bones. (A) Whole-mount Alcian Blue and Alizarin Red stained skeletal preparations of P1 *Vegfa^{+/+}; Osx-Cre:GFP* and *Vegfa^{-/-}; Osx-Cre:GFP* mice. (B) Histology of P1 femurs of *Vegfa^{+/+}; Osx-Cre:GFP* and *Vegfa^{-/-}; Osx-Cre:GFP* mice by H&E (scale bar: 200 μ m), von Kossa and Trap staining, *Osx/GFP⁺* expression and anti-CD31 staining (scale bar: 100 μ m). The bracket indicates increased thickness of the growth plate region in mutant compared with control femur. The boxes indicate central regions of the primary ossification center (POC), similar to those shown at higher magnification beneath. Bar charts show quantification of bone diameter, mineralization, Trap staining and *Osx/GFP* expression in femur sections of P1 *Vegfa^{+/+}; Osx-Cre:GFP* ($n=3$) and *Vegfa^{-/-}; Osx-Cre:GFP* ($n=3$) mice. Mean \pm s.d. *P<0.05 or *P<0.01 for comparison between genotypes. (C) *In situ* hybridization on femur sections of P1 *Vegfa^{+/+}; Osx-Cre:GFP* and *Vegfa^{-/-}; Osx-Cre:GFP* mice for *Osx* (red) and *Col1a1* (turquoise) (scale bars: 200 μ m, top; 100 μ m, bottom). To the right are magnified views of *Osx* and *Col1a1* expression in femoral POC.

Vegfa stimulates perichondrial vascularity and osteoblast differentiation

To address mechanisms underlying reduced numbers of *Osx*⁺ cells in P1 femurs, we analyzed *Osx/GFP⁺* cells during formation of POCs. E15.5 tibia of mutant mice showed markedly decreased numbers of *Osx/GFP⁺* cells (43.2 \pm 4.9 per defined region of perichondrium and POC) compared with WT (56.4 \pm 6.5) mice

(Fig. 2A). The area of anti-CD31 staining in POCs as a percentage of stained areas in perichondrium and POC combined was 0.6 \pm 0.5% for mutant compared with 23.4 \pm 5.5% for controls. Likewise, the area of Trap staining for osteoclasts in POCs as a percentage of total staining in perichondrium and POC combined was 27.7 \pm 8.5% for mutant compared with 53.6 \pm 17.2% in controls. This is consistent with published findings that

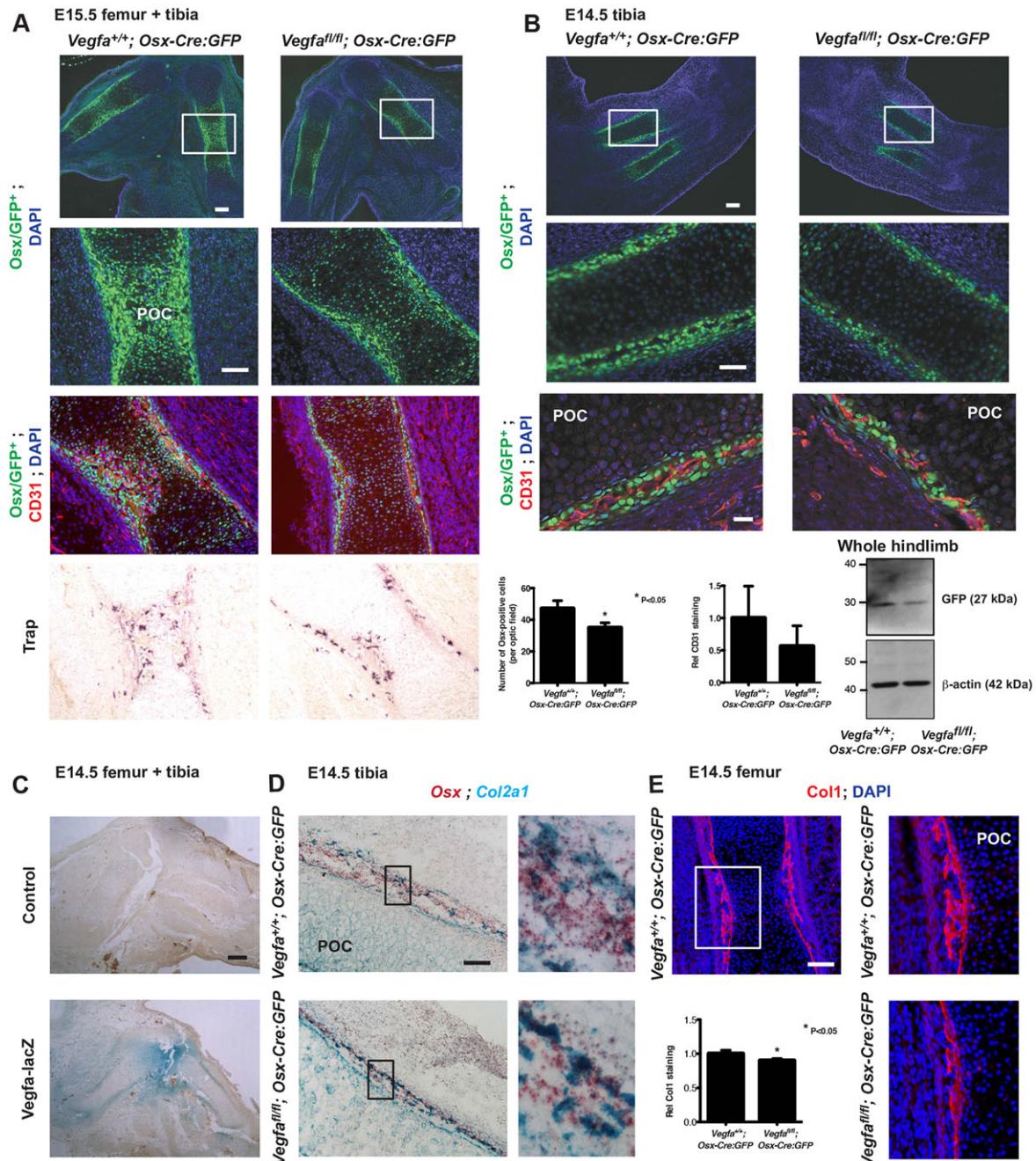


Fig. 2. Loss of Vegfa expression in osteolineage cells decreases perichondrial vascularity and osteoblast precursor numbers. (A) Osx/GFP⁺ cells (green) in E15.5 Vegfa^{+/+}; Osx-Cre:GFP (top left) and Vegfa^{fl/fl}; Osx-Cre:GFP (top right) femur and tibia sections (scale bar: 200 µm). The boxed femoral areas are magnified beneath (scale bar: 100 µm), showing anti-CD31 and Trap staining. (B) Osx/GFP⁺ cells in E14.5 Vegfa^{+/+}; Osx-Cre:GFP (top left) and Vegfa^{fl/fl}; Osx-Cre:GFP (top right) tibia and fibula sections (scale bar: 200 µm). Beneath are magnified views of the tibia areas boxed above (scale bar: 50 µm) and anti-CD31 staining of perichondrial areas (scale bar: 25 µm). Bar charts show quantification of Osx/GFP⁺ cells in the optic field and quantification of anti-CD31 staining in perichondrial areas of E14.5 tibia of Vegfa^{+/+}; Osx-Cre:GFP ($n=3$) and Vegfa^{fl/fl}; Osx-Cre:GFP ($n=3$) mice. Values represent the mean number of Osx/GFP⁺ cells per optic field or mean relative anti-CD31 staining \pm s.d. * $P<0.05$ for comparison between genotypes. The western blot is of GFP in E14.5 Vegfa^{+/+}; Osx-Cre:GFP and Vegfa^{fl/fl}; Osx-Cre:GFP whole hindlimb lysates. β -actin provides a loading control. (C) LacZ staining indicating Vegfa-expressing cells in femur and tibia sections from E14.5 Vegfa-lacZ mice as compared with WT control tissue (scale bar: 500 µm). (D) In situ hybridization on tibia sections of E14.5 Vegfa^{+/+}; Osx-Cre:GFP and Vegfa^{fl/fl}; Osx-Cre:GFP mice for Osx (red) and Col2a1 (turquoise) (scale bar: 50 µm). To the right are magnified views of the boxed perichondrial areas showing cells co-expressing Osx and Col2a1. (E) (Top left) Anti-Col1 staining of femur sections from E14.5 Vegfa^{+/+}; Osx-Cre:GFP mice (scale bar: 100 µm). (Bottom left) Quantification of Col1 staining of femur sections from Vegfa^{+/+}; Osx-Cre:GFP ($n=3$) and Vegfa^{fl/fl}; Osx-Cre:GFP ($n=3$) mice. Values represent mean relative staining area \pm s.d. * $P<0.05$ for comparison between genotypes. (Right) Magnified views of the boxed area of WT control and Vegfa^{fl/fl}; Osx-Cre:GFP mice.

Vegfa derived from Osx⁺ prehypertrophic chondrocytes attracts these cells into POCs (Maes et al., 2010b; Zelzer et al., 2002, 2004).

Loss of Vegfa expression in Osx⁺ cells resulted in decreased numbers of Osx/GFP⁺ cells in the perichondrial region of E14.5 tibia and reduced GFP levels in the whole hindlimb as assessed by

western blotting. Area of anti-CD31 staining indicated reduced, but not significant, differences in the perichondrium of mutant tibia (Fig. 2B), whereas the area of anti-CD34 staining, a marker of hematopoietic/endothelial cells, was reduced (supplementary material Fig. S2A). This suggests that reduced perichondrial vascularity might contribute to the decreased numbers of Osx/GFP⁺ cells. BrdU and TUNEL stainings confirmed that proliferating and apoptotic cell numbers were unaffected in E14.5 *Vegfa^{fl/fl}; Osx-Cre:GFP* mice (supplementary material Fig. S2B,C).

X-gal staining of hindlimbs of E14.5 *Vegfa*-lacZ mice indicated that perichondrial cells of tibia and femur express *Vegfa* (Fig. 2C; supplementary material Fig. S2D). No false-positive cells were observed in the control, as osteoclasts are hardly present (supplementary material Fig. S2E). At E14.5, the perichondrium contains specific pools of osteoblast lineage cells, including osteochondroprogenitors ($Col2^+$), osteoblast precursors (Osx^+) and mature osteoblasts ($Col1^+$) (Maes et al., 2010b). *In situ* hybridization confirmed co-expression of *Osx* and *Col2* (*Col2a1*) in perichondrial cells (Fig. 2D), and perichondrial osteochondroprogenitors and their progeny were present in mice carrying *Tomato* and *Col2-Cre* transgenes (supplementary material Fig. S2F).

Next, we compared mice carrying floxed *Vegfa* alleles and *Osx-Cre:GFP* or *Col2-Cre* transgenes. Loss of *Vegfa* expression in *Osx/GFP⁺* cells in *Vegfa^{fl/fl}; Osx-Cre:GFP* mice was confirmed by *in situ* hybridization (supplementary material Fig. S2G). *Osx* expression was reduced and *Col2* expression appeared increased in tibia sections of E14.5 mutant mice (Fig. 2D), suggesting reduced differentiation of $Col2^+$ osteochondroprogenitors into Osx^+ cells. Probing sections for *Col1* mRNA (supplementary material Fig. S2H) or *Col1* protein revealed that loss of *Vegfa* in *Osx/GFP⁺* cells or $Col2^+$ cells resulted in slightly reduced *Col1* expression in perichondrial areas (Fig. 2E; supplementary material Fig. S2I). Thus, *Vegfa* produced by $Col2^+$ and Osx^+ osteoprogenitors appears to primarily function as a stimulator of osteolineage differentiation.

Vegfa stimulates Vegfr2 signaling and β -catenin expression

Most effects of *Vegfa* are mediated by *Vegfr1* (Flt1 – Mouse Genome Informatics) and *Vegfr2* (Kdr – Mouse Genome Informatics; also known as Flk1) (Ferrara et al., 2003). *Vegfr2* regulates chemotaxis, mitogenesis and cytoskeletal reorganization (Carmeliet and Collen, 1999); *Vegfr1* primarily acts as a decoy receptor, although its function depends on the developmental stage (Ferrara et al., 2003). Overexpression of *Vegfa* in $Col2^+$ osteochondroprogenitors enhances bone mass by mechanisms involving *Vegfr2* and β -catenin pathways (Maes et al., 2010a). Staining tibia sections of E14.5 *Vegfa^{+/+}; Osx-Cre:GFP* mice with anti-*Vegfr2* indicated abundant *Vegfr2* expression in perichondrial *Osx/GFP⁺* and likely $Col2^+$ cells (Fig. 3A).

Conditional deletion of *Vegfr2* in *Vegfr2^{fl/fl}; Osx-Cre:GFP* mice resulted in reduced numbers of *Osx/GFP⁺* cells (Fig. 3A). *Col1* levels appeared reduced in the perichondrium of *Vegfr2^{fl/fl}; Osx-Cre:GFP* (Fig. 3B) and *Vegfr2^{fl/fl}; Col2-Cre:GFP* mice (supplementary material Fig. S3A). Perichondrial regions of E14.5 femurs from *Vegfr2^{fl/fl}; Tomato; Col2-Cre* and *Vegfr2^{+/+}; Tomato; Col2-Cre* mice had similar numbers of *Tomato*-positive cells (supplementary material Fig. S3B). Therefore, *Vegfa*-dependent control of differentiation early in the osteoblast lineage appears to be mediated by *Vegfr2*. *Vegfr1* is also abundantly expressed in perichondrial cells; however, conditional deletion of

this receptor in osteoblast lineage cells had no clear effect on the numbers of *Osx/GFP⁺* cells and *Col1* levels in the perichondrium of E14.5 tibia (supplementary material Fig. S3C,D). Newborn *Vegfr2^{fl/fl}; Osx-Cre:GFP* and *Vegfr1^{fl/fl}; Osx-Cre:GFP* mice had no apparent defects compared with their littermate controls (supplementary material Fig. S3E).

Phosphorylation of *Vegfr2*, *Akt* and *Gsk3 β* in hindlimb lysates of E14.5 *Vegfa^{fl/fl}; Osx-Cre:GFP* was decreased, whereas *Mapk* phosphorylation was unaffected (Fig. 3C). Furthermore, loss of *Vegfa* or *Vegfr2* in *Osx⁺* cells resulted in reduced β -catenin expression. Anti- β -catenin staining of tibia sections confirmed reduced β -catenin expression in perichondrial cells (Fig. 3D). Thus, *Vegfa*-induced *Vegfr2* signaling and possibly also the β -catenin pathway regulate osteoblast differentiation in the perichondrium of developing bones.

Vegfa stimulates Ihh expression and represses Notch2 levels

Apart from Wnt/ β -catenin, several other factors regulate endochondral osteoblast differentiation, including *Bmps*, *Fgfs*, *PthrP*, *Ihh* (Colnot et al., 2005; Hu et al., 2005; Razzaque et al., 2005) and *Notch2* (Hilton et al., 2008). To assess possible roles in *Vegfa*-dependent control of differentiation, we analyzed *Ihh* and *Notch2* expression in hindlimb lysates from E14.5 *Vegfa^{fl/fl}; Osx-Cre:GFP* and *Vegfr2^{fl/fl}; Osx-Cre:GFP* mice. Loss of *Vegfa* or *Vegfr2* in *Osx⁺* cells resulted in decreased *Ihh* expression but increased *Notch2* (Fig. 4A). *Ihh* is expressed in prehypertrophic chondrocytes and cells located in perichondrium during endochondral ossification (Hu et al., 2005; Mak et al., 2006). *In situ* hybridization of tibia sections from *Vegfa^{fl/fl}; Osx-Cre:GFP* and *Vegfa^{+/+}; Osx-Cre:GFP* mice indicated that *Ihh* expression was reduced in prehypertrophic mutant chondrocytes (Fig. 4B), whereas no *Ihh* expression was detected in mutant and control perichondrial cells (supplementary material Fig. S4A). Thus, reduced *Ihh* expression in prehypertrophic chondrocytes might directly affect osteoblast differentiation during perichondrial maturation. Anti-*Notch2* staining of tibia sections showed increased *Notch2* expression in perichondrial cells of mutants (Fig. 4C). These data suggest functional links between *Vegfa/Vegfr2*, *Ihh* and *Notch2* in perichondrial osteoblast differentiation.

To test whether *Vegfa* directly regulates the expression of *Ihh* and *Notch2*, we used hindlimb cells from E13.5 *Vegfa^{fl/fl}* mice for *in vitro* differentiation assays. The majority of *Vegfa*-expressing cells showed nuclear *Osx* expression (Fig. 4Da). Adenoviral Cre-mediated *Vegfa* knockdown was confirmed by measuring cell-associated and secreted *Vegfa* levels (supplementary material Fig. S4Ba). Expression of the early and late osteoblast marker genes *Runx2*, *Osx* (*Sp7*) and *Colla1*, and the mRNA levels of *Ihh* and the *Ihh* target genes *Patched 1* (*Ptch1*), *Gli1* and hedgehog interacting protein (*Hhip*) were reduced upon loss of *Vegfa* expression (Fig. 4Db,c). Expression of *Notch2* and target gene hairy/enhancer-of-split related with YRPW motif 1 (*Hey1*) was unaffected (supplementary material Fig. S4Bb), suggesting the possibility that cell-cell contact *in vivo* is important for regulation of *Notch2* expression by *Vegfa*.

In rescue experiments, *Osx* expression was eliminated in the presence of the hedgehog (*Hh*) antagonist GANT-58, whereas recombinant *Vegfa* had only minor effects and *Runx2* and *Colla1* levels were not affected (Fig. 4Dd). This raises the possibility that *Vegfa* might require one or more co-factors to exert its effect on osteoblast differentiation; alternatively, *Vegfa* functions via intracrine rather than paracrine mechanisms, as described in

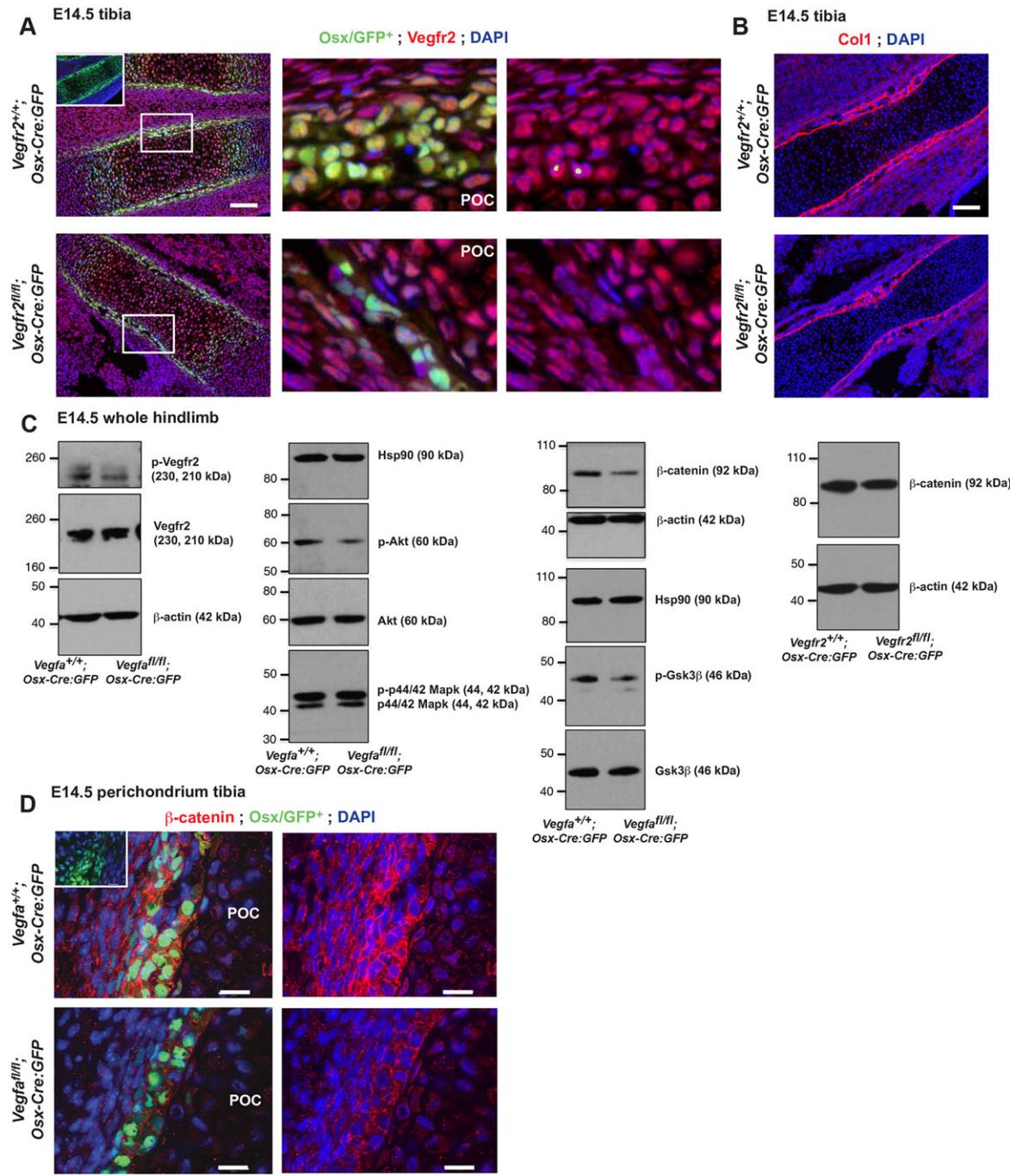


Fig. 3. Loss of Vegfr2 in osteoprogenitors results in reduced osteoblast differentiation and loss of Vegfa/Vegfr2 signaling reduces β-catenin expression in perichondrial cells. (A) (Left) Anti-Vegfr2 staining of tibia sections from E14.5 *Vegfr2^{+/+;} Osx-Cre:GFP* and *Vegfr2^{fl/fl;} Osx-Cre:GFP* mice (scale bar: 100 μm); inset shows non-immune IgG control. Boxed regions are magnified to the right, showing *Osx/GFP⁺* cells (green, middle column) or Vegfr2 staining only (right column). (B) Anti-Col1 staining of tibia sections from E14.5 *Vegfr2^{+/+;} Osx-Cre:GFP* and *Vegfr2^{fl/fl;} Osx-Cre:GFP* mice (scale bar: 100 μm). (C) Western blotting of phospho-Vegfr2 (Tyr1175), phospho-Akt (Ser473), phospho-p44/42 Mapk (Mapk3/1) (Thr202/Tyr204), β-catenin and phospho-Gsk3β (Ser9) protein levels in whole hindlimb lysates from E14.5 *Vegfa^{+/+;} Osx-Cre:GFP*, *Vegfa^{fl/fl;} Osx-Cre:GFP* and *Vegfr2^{fl/fl;} Osx-Cre:GFP* mice; β-actin and Hsp90 provide loading controls. (D) Anti-β-catenin staining of perichondrial area of tibia sections from E14.5 *Vegfa^{+/+;} Osx-Cre:GFP* and *Vegfa^{fl/fl;} Osx-Cre:GFP*; inset shows non-immune IgG control (scale bars: 20 μm). To the right is shown anti-β-catenin staining alone.

adult mice (Liu et al., 2012). We also tested the possible involvement of Gαs (Gnas – Mouse Genome Informatics), which is a modulator of Wnt/β-catenin and Hh signaling activities in mesenchymal progenitors (Regard et al., 2011, 2013). In hindlimb lysates, Gαs expression was not affected in *Vegfa^{fl/fl;} Osx-Cre:GFP* mice (supplementary material Fig. S4Ca). Furthermore, the activity of protein kinase A (Pka), a major

regulator of Hh signaling, was unaffected in these mice (supplementary material Fig. S4Cb).

In summary, our data demonstrate that Vegfa produced by *Osx⁺* precursors regulates blood vessel recruitment and early stages of osteoblast differentiation during perichondrial maturation. Vegfa-dependent effects are mediated by Vegfr2 and mechanisms that include stimulated expression and activity of Ihh, increased

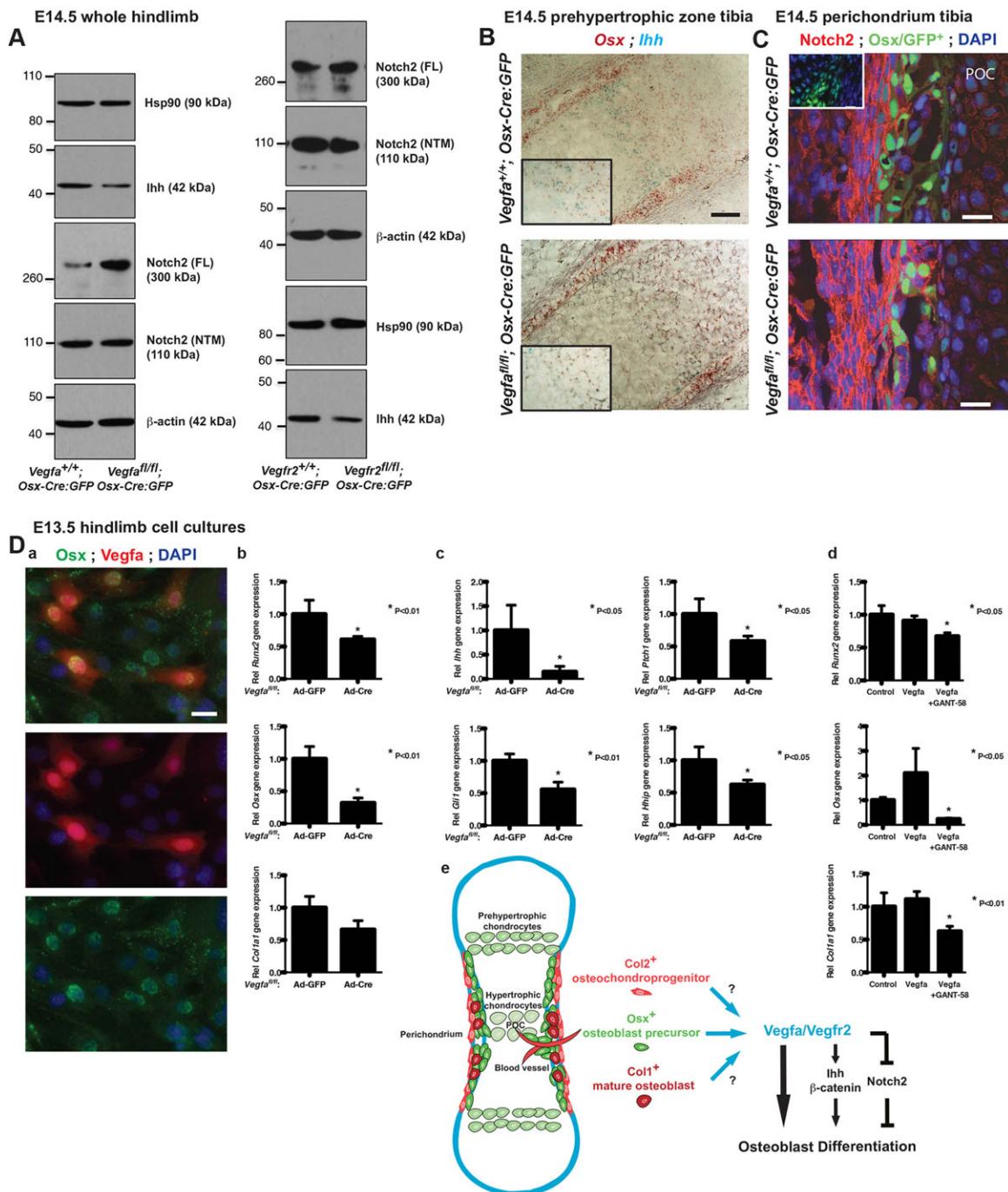


Fig. 4. Vegfa stimulates Ihh/Gli1 signaling and inhibits Notch2 expression in perichondrial cells. (A) Western blotting of Ihh and Notch2 in whole hindlimb lysates from E14.5 *Vegfa/Vegfr2^{+/+}; Osx-Cre:GFP*, *Vegfa^{fl/fl}; Osx-Cre:GFP* and *Vegfr2^{fl/fl}; Osx-Cre:GFP* mice; β -actin and Hsp90 are loading controls. (B) *In situ* hybridization of Osx (red) and Ihh (turquoise) on tibia sections (prehypertrophic zone region) from E14.5 *Vegfa^{+/+}; Osx-Cre:GFP* and *Vegfa^{fl/fl}; Osx-Cre:GFP* mice (scale bars: 50 μ m). Insets are magnified views of prehypertrophic chondrocyte areas. (C) Anti-Notch2 staining of perichondrial area of tibia sections from E14.5 *Vegfa^{+/+}; Osx-Cre:GFP* and *Vegfa^{fl/fl}; Osx-Cre:GFP* mice; inset shows non-immune IgG control (scale bars: 20 μ m). (D) (a) Anti-Vegfa and anti-Osx staining of cultured hindlimb cells from E13.5 *Vegfa^{fl/fl}* embryos (scale bar: 50 μ m). (b,c) Expression, as assessed by qRT-PCR, of *Runx2*, *Osx* (*Sp7*), *Col1a1*, *Ihh*, *Ptch1*, *Gli1* and *Hhip* in lysates of cultured hindlimb cells from E13.5 *Vegfa^{fl/fl}* embryos, treated with adenoviral Cre and induced to differentiate into osteoblasts. Control cells were treated with adenoviral GFP. Relative gene expression levels are normalized to levels of *Gapdh*. Values represent mean relative expression \pm s.d. ($n=3$). (d) Expression (by qRT-PCR) of *Runx2*, *Osx* (*Sp7*) and *Col1a1* in lysates of cultured hindlimb cells from E13.5 *Vegfa^{fl/fl}* embryos, treated with adenoviral Cre and induced to differentiate into osteoblasts in the presence of recombinant Vegfa with or without the Hh antagonist GANT-58, as compared with control cells. Relative gene expression levels were normalized to levels of *Gapdh*. Values represent mean relative expression \pm s.d. ($n=3$). (e) Model summarizing our findings and conclusions. During perichondrial maturation, Col2⁺ osteochondroprogenitors differentiate into Osx⁺ precursors, which either migrate into the POC in response to Vegfa secreted by (pre)hypertrophic chondrocytes or mature into Col1⁺ osteoblasts in the perichondrium. Vegfa derived from perichondrial osteolineage cells controls osteoblast differentiation in a Vegfr2-dependent manner, likely involving Ihh-, β -catenin- and Notch2-dependent pathways.

expression of β -catenin and inhibition of Notch2 (Fig. 4D). Since many factors are crucial for osteoblast differentiation during endochondral bone formation, Vegfa/Vegfr2-dependent regulation of osteoblast differentiation might involve additional mechanisms as well. Furthermore, in addition to an autocrine/paracrine role of Vegfa, changes in perichondrial vascularity indicative of a general developmental delay might also contribute to the effects on osteoblast differentiation.

MATERIALS AND METHODS

Mouse strains

Floxed *Vegfa*, *Vegfr2* (*Flk1*) and *Vegfr1* (*Flt1*) mice were generated at Genentech. 129-*Vegfam1.1Nagy* (Miquerol et al., 1999), *Osx-Cre:GFP* (Rodda and McMahon, 2006) and *Col2-Cre* (Schipani et al., 2001) mice have been described previously. B6.Cg-*Gt(ROSA)26Sortm1(CAG-tdTomato)Hze/J* mice were purchased from Jackson Laboratory. All animal experiments were performed with protocols approved by Harvard Medical Area Standing Committee on Animals and in accordance with US Public Health Service Policy on Humane Care and Use of Laboratory Animals. Further details of mouse strains and genotyping are provided in the supplementary Materials and Methods and Table S1.

Staining, histology and microCT

Alizarin Red and Alcian Blue staining of skeletal structures in P1 mice and microCT analyses on hindlimbs are described in the supplementary Materials and Methods. Frozen sections (7.5 μ m) were prepared for histology, including Hematoxylin and Eosin (H&E), von Kossa and Trap staining, and analyzed with a Nikon 80i upright microscope or a Nikon Ti w/ spinning disk confocal microscope as described in the supplementary Materials and Methods. X-Gal staining to detect *Vegfa-lacZ* expression, and BrdU and TUNEL staining of proliferating and apoptotic cells are detailed in the supplementary Materials and Methods.

Immunohistochemistry (IHC), immunocytochemistry (ICH) and western blotting

IHC on frozen limb sections, ICH on fixed cells in culture chambers and western blotting of protein lysates of E14.5 hindlimbs were carried out with the antibodies and imaging techniques described in the supplementary Materials and Methods.

ELISA

Vegfa protein levels in cell lysates (cell associated) or culture medium (secreted) were assessed and normalized as described in the supplementary Materials and Methods.

RNA *in situ* hybridization and qRT-PCR

Gene expression analyses by RNA *in situ* hybridization and qRT-PCR were carried out using the probes and primers detailed in the supplementary Materials and Methods and Table S2.

Mesenchymal progenitor cell cultures

Hindlimbs of E13.5 embryos were enzymatically digested and cells infected with either Ad-Cre or Ad-GFP followed by osteogenic induction as described in the supplementary Materials and Methods.

Quantifications and statistical analysis

Quantifications of femur diameter, mineralization and cell counts (Osx, Trap, CD31, CD34 and Col1 staining) were performed as described in the supplementary Materials and Methods. Results are presented as mean \pm s.d. and unpaired Student's *t*-tests were used. $P<0.05$ was considered significant.

Acknowledgements

We thank Yulia Pittel for secretarial assistance; Sofiya Plotkina and Karen Cox for technical support; Andras Nagy (Lunenfeld-Tanenbaum Research Institute, Canada) for providing Vegfa-lacZ mice; Napoleone Ferrara (UC San Diego) and

Genentech for providing mice with floxed *Vegfa*, *Flk1* and *Flt1* alleles; Beate Lanske (HSDM) for providing *Col2-Cre* mice; and Valerie Salazar (HSDM) for advice on X-gal staining. We acknowledge services of the MicroCT Core at HSDM and Nikon Imaging Center at HMS.

Competing interests

The authors declare no competing or financial interests.

Author contributions

X.D., B.R.O. and A.D.B. designed experiments and interpreted results. X.D., Y.M., Y.L., C.N. and A.D.B. performed the experiments. X.D., B.R.O. and A.D.B. wrote the manuscript.

Funding

This work was supported by the National Institutes of Health [grants AR36819, AR36820 and AR48564 to B.R.O.] and partially supported by an HSDM Dean's Scholarship (to A.D.B.). Deposited in PMC for release after 12 months.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.117952/-DC1>

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