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VEGF stimulates intramembranous bone formation during craniofacial skeletal development

Xuchen Duan, Seth R. Bradbury, Bjorn R. Olsen, and Agnes D. Berendsen

, Harvard School of Dental Medicine, Boston, MA 02115, USA

Abstract

Deficiency of vascular endothelial growth factor A (VEGF) has been associated with severe craniofacial anomalies in both humans and mice. Cranial neural crest cell (NCC)-derived VEGF regulates proliferation, vascularization and ossification of cartilage and membranous bone. However, the function of VEGF derived from specific subpopulations of NCCs in controlling unique aspects of craniofacial morphogenesis is not clear. In this study a conditional knockdown strategy was used to genetically delete *Vegfa* expression in Osterix (Osx) and collagen II (Col2)-expressing NCC descendants. No major defects in calvaria and mandibular morphogenesis were observed upon knockdown of VEGF in the Col2⁺ cell population. In contrast, loss of VEGF in Osx⁺ osteoblast progenitor cells led to reduced ossification of calvarial and mandibular bones without affecting the formation of cartilage templates in newborn mice. The early stages of ossification in the developing jaw revealed decreased initial mineralization levels and a reduced thickness of the collagen I (Col1)-positive bone template upon loss of VEGF in Osx⁺ precursors. Increased numbers of proliferating cells were detected within the jaw mesenchyme of mutant embryos. Explant culture assays revealed that mandibular osteogenesis occurred independently of paracrine VEGF action and vascular development. Reduced VEGF expression in mandibles coincided with increased phospho-Smad1/5 (P-Smad1/5) levels and bone morphogenetic protein 2 (*Bmp2*) expression in the jaw mesenchyme. We conclude that VEGF derived from Osx⁺ osteoblast progenitor cells is required for optimal ossification of developing mandibular bones and modulates mechanisms controlling BMP-dependent specification and expansion of the jaw mesenchyme.

Keywords

VEGF; Craniofacial ossification; Bone development; Calvaria; Mandible; Osterix

Correspondence to Agnes D. Berendsen: 188 Longwood Ave, REB 413, Boston, MA 02115, USA. ; Email: Agnes_Berendsen@hsdm.harvard.edu.

Author contributions

X.D, S.B., B.R.O and A.D.B designed experiments and interpreted results. X.D, S.B., and A.D.B performed the experiments. X.D, B.R.O and A.D.B wrote the manuscript.

Competing interests

The authors declare no competing financial interests.

1. Introduction

Developmental abnormalities in the growth of bones of the skull and face cause craniofacial defects such as unfused cranial sutures, mandibular hypoplasia, and cleft palate.

Mechanisms underlying the etiology of such defects are incompletely understood and treatment options are limited. The craniofacial skeleton predominantly develops through intramembranous ossification involving NCC-derived progenitor cells [1–3]. The migration and differentiation of NCCs during morphogenesis of cartilage and membranous bone are tightly regulated by signals derived from various cell types.

VEGF is abundantly expressed by NCCs during craniofacial development [4]. Mice lacking the secreted isoform VEGF164 show craniofacial defects including cleft palate, unfused cranial sutures, and shorter jaws. Similar calvarial and mandibular malformations are observed upon conditional deletion of VEGF in NCCs [5,6]. Such defects were attributed to changes in VEGF-dependent proliferation, vascularization, and ossification during cartilage and bone formation. VEGF was also reported to regulate calvarial ossification [7] and maxillary and palatal mesenchyme [6] independent of vascular development. Furthermore, VEGF stimulates BMP2 expression prior to and during ossification of palatal shelves and maxilla, adding another layer of complexity to the function of VEGF during cartilage and membranous bone formation.

NCCs give rise to a variety of cell types and tissues during embryonic development, including cells that form the intramembranous bone, cartilage, and teeth [8,9]. Osteoblast progenitor cells and their progeny express the transcription factor *Osx*, which can also be detected in mesenchymal cells of the tooth germ but not in the epithelial tissue [10]. At embryonic day 15.5 (E15.5) abundant *Osx* expression is associated with bone formation while Meckel's cartilage lacks *Osx* expression. About 35% of the *Osx*⁺ precursors were previously reported to also be *Col2* positive prior to becoming mature osteoblasts in developing dermal bones [11]. Furthermore, *Col2*-expressing cells are abundantly detected in the skull base, nasal region and Meckel's cartilage during craniofacial skeletal development [12]. At the initial stages of craniofacial ossification *Vegfa* expression was reported to be particularly high in the mesenchyme giving rise to the calvaria and mandible with low expression in the cartilage structures [5]. However, the contribution of VEGF derived from different subpopulations of NCCs, including *Osx*⁺ osteoblast progenitor cells and *Col2*⁺ cells, in the regulation of craniofacial skeletal development is not clear.

In this study, we show that VEGF derived from *Osx*⁺ osteoblast progenitor cells is required for optimal intramembranous bone formation in the developing jaw. The VEGF-dependent mechanisms appear to be independent of paracrine VEGF function and vascular development and likely involve the BMP-dependent specification and proliferation of cells within the jaw mesenchyme during mandibular osteogenesis.

2. Results

2.1. *Osx*- and *Col2*-expressing cells are distinct populations of VEGF-producing cells during cartilage and intramembranous bone formation

To assess the function of VEGF expressed by different subpopulations of NCCs, we first explored the presence of *Osx*-expressing osteoblast progenitor cells and *Col2*-expressing cells during craniofacial development. *In situ* hybridization on frontal sections of the heads of E14.5 and E16.5 wild-type (WT) (*Vegfa*^{+/+}; *Osx-Cre:GFP*) embryos showed expression of *Osx* and *Col2* in distinct areas of developing calvaria and mandibles (Fig. 1A). *Osx*-expressing mesenchymal cells were detected surrounding the *Col2*⁺ Meckel's cartilage with a substantial number of mesenchymal cells co-expressing *Osx* and *Col2*. Such *Col2*⁺ osteoblastic progenitors were previously reported giving rise to about 35% of mature osteoblasts in developing mandibles [11]. Prior to generating mice with conditional loss of *Vegfa* alleles in *Osx*- and *Col2*-expressing cells we confirmed that the expression of *Osx-Cre:GFP* and *Col2-Cre* transgenes was similar to the endogenous *Osx* and *Col2* expression profiles during craniofacial skeletal development (Fig. 1B). Indeed, the location of *Osx*/GFP-positive cells was overlapping with the endogenous *Osx* expression patterns in calvarial and mandibular areas of E14.5 and E16.5 *Vegfa*^{+/+}; *Osx-Cre:GFP* embryos (Fig. 1B). *Col2*-expressing cells and their progeny, assessed by *Col2-Cre*-induced Tomato expression in craniofacial areas of E14.5 and E16.5 *Vegfa*^{+/+}; *Tomato*; *Col2-Cre* embryos, were abundantly detected in cartilaginous structures as well as in mandibular and other craniofacial regions (Fig. 1B). Mesenchymal cells located in both developing mandible and calvaria showed significant levels of *Vegfa* expression as assessed by *in situ* hybridization on frontal sections of the heads of E14.5 *Vegfa*^{+/+}; *Osx-Cre:GFP* embryos (Fig. 1C). In contrast, *Vegfa* expression appeared rather low in chondrocytes of the Meckel's cartilage. Conditional deletion of *Vegfa* in *Osx*⁺ precursors was confirmed by *in situ* hybridization on frontal sections of the heads of E14.5 *Vegfa*^{fl/fl}; *Osx-Cre:GFP* mice (Fig. 1C).

2.2. VEGF stimulates intramembranous bone formation during craniofacial skeletal development

The skulls of newborn *Vegfa*^{fl/fl}; *Osx-Cre:GFP* mice were slightly smaller and showed significantly enlarged frontal fontanelle as assessed by measurement of the width of the sagittal suture compared with control *Vegfa*^{+/+}; *Osx-Cre:GFP* mice (Fig. 2A). In addition, mandibular ossification was significantly reduced in mutant mice while jaw length and palatogenesis appeared unaffected (Fig. 2A). Histological analysis of frontal sections of the heads of *Vegfa*^{fl/fl}; *Osx-Cre:GFP* mice revealed thinner calvaria and mandibular structures as assessed by Von Kossa and anti-Col1 staining with fewer Trap-positive osteoclasts compared with control mice (Fig. 1B). Surprisingly, loss of *Vegfa* in *Col2*⁺ cells led to no major ossification defects in newborn *Vegfa*^{fl/fl}; *Col2-Cre* mice (Fig. 3A, 3B) although the overall size of the skull and mandible appeared slightly reduced in a proportional manner compared to control *Vegfa*^{+/+}; *Col2-Cre* mice (Fig. 3A). Von Kossa staining of frontal sections of the heads of *Vegfa*^{fl/fl}; *Col2-Cre* mice showed a reduced size of the cartilage primordium of orbito-sphenoid bone (Fig. 3B). As expected, the skeletal elements obtained from WT control mice carrying the *Osx-Cre:GFP* transgene (Fig. 2A) showed slightly diminished

ossification compared to control mice carrying the Col2-Cre transgene (Fig. 3A) as *Osx-Cre:GFP* was previously reported to cause craniofacial bone development defects [13].

2.3. VEGF derived from *Osx*-expressing cells enhances initial mandible ossification independent of vascular development

To address the underlying mechanisms by which VEGF derived from *Osx*⁺ precursors regulates membranous bone formation, we focused on early jaw morphogenesis. Recent reports highlighted a role for NCC-derived VEGF in embryonic jaw extension [5] and palatogenesis [6]. At E14.5 the Meckel's cartilage has gained its mature shape while osteogenesis of the mandible is in the initial stages [5]. Although the length of the Meckel's cartilage appeared unaffected in E15.5 *Vegfa*^{fl/fl}; *Osx-Cre:GFP* mice, mandibular ossification was already reduced compared with littermate *Vegfa*^{+/+}; *Osx-Cre:GFP* controls (Fig. 4A). *In situ* hybridization on frontal sections of the heads of E16.5 embryos showed that the ratio of mesenchymal cells expressing *Osx* and *Col2* was not affected in *Vegfa*^{fl/fl}; *Osx-Cre:GFP* mice (Fig. 4B). Additional histological analysis of frontal sections of E16.5 heads indicated that *Osx*/GFP expression and Col1-stained areas outlining mandibular thickness was reduced in mutant *Vegfa*^{fl/fl}; *Osx-Cre:GFP* compared with control embryos (Fig. 4B). Apoptosis of *Osx*⁺ precursors as assessed by TUNEL staining was not affected in developing mandibles of E16.5 *Vegfa*^{fl/fl}; *Osx-Cre:GFP* embryos (Fig. 5A). However, anti-Ki67 staining revealed increased numbers of proliferating cells within the jaw mesenchyme of mutant embryos (2.45 ± 1.38 times compared to controls) (Fig. 5B). Since NCC-derived VEGF was previously reported to regulate the growth of the mandibular artery leading to extension of the Meckel's cartilage [5], we assessed to what extent VEGF derived from *Osx*⁺ precursors affected vascularization of developing mandibles. Anti-CD31 staining indicated that the mandibular artery and the presence of blood vessels within the jaw mesenchyme were not affected in E16.5 *Vegfa*^{fl/fl}; *Osx-Cre:GFP* embryos (Fig. 5C), indicating that defective intramembranous bone formation upon loss of VEGF in *Osx*⁺ precursors is largely independent of vascular development.

2.4. VEGF-dependent mandibular ossification is not mediated by paracrine VEGF/VEGFR2 signaling in *Osx*⁺ precursors

VEGF was previously reported to stimulate intramembranous ossification during calvarial explant culture *in vitro* [7]. To assess whether mandibular osteogenesis is regulated by paracrine VEGF signaling, we performed explant cultures with mandibles obtained from E15.5 WT mice (Fig. 6Aa). Dissected mandibles were grown in the presence of 10 ng/ml VEGF164 for 7 days followed by analysis of their ossification levels compared to untreated controls (Fig. 6Ab). A substantial increase in the Alizarin Red-stained areas surrounding the Alcian Blue-positive Meckel's cartilage was detected in both VEGF treated and control mandibles (Fig. 6Ab). However, VEGF treatment had no significant effect on the ossification levels (Fig. 6Ac), suggesting that paracrine VEGF function did not regulate mandibular bone formation. To further address the role of paracrine VEGF signaling in *Osx*⁺ precursors we assessed the expression of the major VEGF receptor involved in VEGF signaling, VEGFR2. *In situ* hybridization indicated that mRNA levels for VEGFR2 were detected in the vascular endothelium but at rather low levels in cells of the jaw mesenchyme of E14.5 and E16.5 *Vegfa*^{+/+}; *Osx-Cre:GFP* embryos (Fig. 6B). Furthermore, loss of

VEGFR2 in *Osx*⁺ precursors did not result in obvious skeletal defects in newborn *Vegfr2*^{fl/fl}, *Osx-Cre:GFP* mice compared to control *Vegfr2*^{+/+}; *Osx-Cre:GFP* mice (Fig. 6C). To confirm that mandibular ossification is not regulated by paracrine VEGF signaling we used adenoviral Cre recombinase to knockdown VEGF in dissected mandibles obtained from E15.5 *Vegfa*^{fl/fl} mice (Fig. 7Aa). VEGF expression was reduced about 40% in protein lysates obtained from adenoviral Cre-treated mandibles compared to the adenoviral GFP-infected control (data not shown). Western blotting of mandibular protein lysates showed robust GFP expression in control mandibles infected with adenoviral GFP and reduced Col1 expression was detected upon VEGF knockdown (Fig. 7Ab). Treatment of mandibles with VEGF164 did not affect intramembranous ossification during mandibular explant culture (Fig. 7Ac). However, the relative levels of mandibular ossification were reduced about 34% compared with controls upon VEGF knockdown (data not shown); this roughly correlates with a 40% decrease in VEGF protein levels. We then addressed the possibility that VEGF-dependent control of intramembranous bone formation involves the regulation of additional signaling pathways. The timing of bone differentiation in mandibular mesenchyme is regulated by BMP signaling pathways [14–16] and VEGF was previously reported to stimulate the expression of BMP2 in NCCs [6]. Furthermore, Wnt/β-catenin is required for craniofacial development [17] while *Ihh* and PTHrP have been identified as negative regulators of osteoblast differentiation during dermal bone formation [11]. Western blotting of mandibular protein lysates upon knockdown of VEGF in jaw mesenchyme revealed increased levels of the downstream BMP mediators P-Smad1/5 while no major differences in the expression of β-catenin or *Ihh* proteins were detected (Fig. 7Ad). Furthermore, *in situ* hybridization on frontal sections of heads of E14.5 *Vegfa*^{fl/fl}, *Osx-Cre:GFP* embryos indicated that loss of VEGF in *Osx*⁺ precursors resulted in increased *Bmp2* expression within jaw mesenchyme during mandibular osteogenesis (Fig. 7B).

3. Discussion

Craniofacial anomalies arise from developmental defects in the formation of calvarial and mandibular bones. Elucidating the mechanisms of chondrogenesis and osteogenesis during craniofacial skeletal development is essential in order to enable innovation in therapeutic strategies for treatment of such craniofacial disorders. We have shown here that VEGF derived from the *Osx*⁺ osteoblast progenitor cell population is required for jaw mesenchyme to promote intramembranous bone formation.

VEGF is abundantly expressed by NCCs and plays multiple roles in regulating complex morphogenetic events during craniofacial skeletal development [5,6]. Not surprisingly, mouse models in which VEGF is specifically deleted in NCCs show dramatic craniofacial phenotypes, including bone hypoplasia of the cranium and mandible, cleft lip and palate, reduced ossification of the premaxillary and frontal bones, and changes in size and shape of the Meckel's cartilage resulting in shorter and misshapen mandibles. Defects upon loss of VEGF in NCCs arise at a developmental point after NCC specification and migration into the mandibular primordium (pharyngeal arch 1) and the anomalies were mainly attributed to inadequate vessel growth and arterial instability in the jaw [5]. To elucidate the role of VEGF derived from specific subpopulations of NCCs during craniofacial skeletal development, we conditionally deleted VEGF in *Osx*⁺ or *Col2*⁺ NCC descendants.

Remarkably, loss of VEGF in *Osx*⁺ precursors but not in the more abundant *Col2*-expressing cell population resulted in mild defects in the formation of craniofacial skeletal elements independent of vascular development. The lack of a phenotype upon deletion of VEGF using the *Col2*-Cre transgene in our studies was rather unexpected. We found that lineage tracing of *Col2*⁺ cells using the Tomato Cre reporter transgene indicated abundant *Col2*⁺ cells and their progeny at different stages during craniofacial skeletal development. Limited VEGF expression in these cells or, alternatively, suboptimal activity of Cre recombinase in *Col2*-expressing cells resulting in a partial knockdown of VEGF expression may explain the apparently normal development of the craniofacial elements upon conditional deletion of VEGF using the *Col2*-Cre transgene.

Our data suggest that VEGF may function in an autocrine manner in *Osx*⁺ precursors as it regulates the specification and expansion of mesenchymal cells in the jaw. Such a role for VEGF is supported by the results obtained from the explant culture assays, which revealed that paracrine VEGF did not affect mandibular osteogenesis *in vitro* (Fig. 8). Furthermore, loss of VEGFR2 in *Osx*⁺ precursors resulted in no major defects in craniofacial skeletal development. In contrast, paracrine VEGF was previously reported to increase osteoblast activity during calvarial growth *in vitro* [7]. Although both calvarial and mandibular bones develop as a result of intramembranous ossification, the mechanisms involved may vary amongst the individual membranous skeletal elements [18]. We propose that paracrine VEGF signaling is indispensable for craniofacial skeletogenesis mediated by vascular development, including regulation of the growth of the mandibular artery followed by embryonic jaw extension (Fig. 8) [5].

The VEGF-dependent control of mandibular osteogenesis likely involves mechanisms at the stage of preosteoblast specification and expansion. Loss of VEGF in *Osx*⁺ precursors but not in *Col2*⁺ osteochondroprogenitors was characterized by increased cell proliferation within the jaw mesenchyme. The thickness of the differentiating mandibular template was reduced but the ratio between *Osx*⁺ precursors and *Col2*⁺ osteochondroprogenitors appeared unaffected. Since the initial specification of NCCs into chondrocytes and preosteoblasts is primarily controlled by BMP signaling, we addressed the possibility that loss of VEGF in *Osx*⁺ precursors could modulate this pathway. The levels of downstream mediators P-Smad1/5 were increased upon reduction of VEGF levels in jaw mesenchyme and levels of *Bmp2* expression appeared to be increased as well. In conclusion, VEGF derived from *Osx*⁺ precursors located in the jaw mesenchyme is required for optimal intramembranous bone formation. We propose that VEGF regulates osteogenesis in an autocrine manner independent of paracrine VEGF function and vascular development. The mechanisms of VEGF action likely involve the BMP-dependent specification and proliferation of cells within the jaw mesenchyme during craniofacial skeletal development.

4. Materials and methods

4.1. Mouse strains

Floxed *Vegfa* and *Vegfr2* (*Flk1*) mice used in this study were generated at Genentech. B6.Cg-*Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J* mice were purchased from Jackson Laboratory. *Osx*-Cre:GFP [19] mice and *Col2*-Cre [20] mice have been described

previously. Embryos were collected from timed pregnancies, and E0.5 was defined as the day that the male was separated from the female after the overnight mating. Genomic DNA, isolated using standard protocols from portions of mouse tails, was used for genotyping. All primers are listed below. All animal experiments were performed with protocols approved by Harvard Medical Area Standing Committee on Animals in accordance with U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals.

4.2. Primer sequences used for genotyping

	Forward primer	Reverse primer	Product
Generic-Cre	5'-GATGAGGTTTCGCAAGAACCTG-3'	5'-TGAACGAACCTGGTCGAAATC-3'	~350 bp
VEGFA	5'-CCTGGCCCTCAAGTACACCTT-3'	5'-TCCGTACGACGCATTCTAG-3'	~150 bp
VEGFR2	5'-GACTTGGTTCATCAGGCTAG-3'	5'-GACGCTGTTAAGCTGCTACAC-3'	~230 bp
Rosa wildtype	5'-AAGGGAGCTGCAGTGGAGTA-3'	5'-CCGAAAATCTGTGGGAAGTC-3'	~297 bp
Rosa Tomato mutant	5'-CTGTTCTCTGACGGCATGG-3'	5'-GGCATTAAAGCAGCGTATCC-3'	~169 bp

4.3. Skeletal preparations and quantification of mandibular ossification, mandibular length, width of sagittal suture, and mandibular thickness

Bones and cartilage in heads of E15.5 and newborn mice (P1) were visualized by Alizarin Red S and Alcian Blue (Sigma-Aldrich) staining. Skeletal samples were cleared in potassium hydroxide (1%)/glycerol (20%) solution, and stored and photographed in glycerol.

Mandibular ossification, mandibular length, and width of sagittal suture were measured in images of whole mount mandibles and calvaria obtained from newborn mice using ImageJ program. Mandibular ossification was determined as the area of positive Alizarin Red staining in the mandible, mandibular length was defined as the linear distance between the anterior end and posterior end of the mandible. The minimal distance between the parietal bones represents the width of the sagittal suture. Mandibular thickness was measured in mandibular sections of E16.5 embryos using ImageJ program. Seven central-cut sections were analyzed per bone. Mandibular thickness was defined as the distance of the mandibular template across the center of the Meckel's cartilage. All the measurements are presented as relative values of control measurements.

4.4. Histology

For histological analysis, heads of embryos and newborn mice were fixed in 4% paraformaldehyde (PFA) at 4 °C overnight, infiltrated with a series of sucrose and embedded in OCT (Tissue-Tek®) compound (Sakura Finetek USA, Inc., Torrance, CA) for cryostat sectioning. Frozen sections (7.5 µm) were subjected to hematoxylin and eosin (H&E) staining, Von Kossa staining and tartrate-resistant acid phosphatase (Trap) staining as previously described [21]. Photographs of histological sections were taken with a Nikon 80i Upright microscope using NIS-Elements AR3.1 software.

4.5. Double-labeling RNA *in situ* hybridization

Double-labeling RNA *in situ* hybridization was performed on frozen sections of heads using the RNAscope 2-plex Detection Kit (Chromogenic) as described in manufacturer's instructions (Advanced Cell Diagnostics). RNAscope probes used include: Sp7 (NM_130458.3, region 837-2230), which was detected using the Fast Red detection reagent, and Col2a1 (NM_001113515.2, region 729-2036), Vegfa (NM_001025257.3, region 946-2156), Kdr (Vegfr2) (NM_010612.2, region 1766-2673) and Bmp2 (NM_007553.3, region 872-2039), which were detected using the Green detection reagent.

4.6. Immunohistochemistry (IHC)

For IHC, frozen sections of heads were stained with Collagen I (1:100; ab21286; Abcam), Ki67 (1:500; ab16667; Abcam) and CD31 (1:100; ab28364; Abcam) primary antibodies and Alexa Fluor-conjugated secondary antibody (1:200; Invitrogen), according to Cell Signaling Technology protocols. Control sections incubated with control rabbit IgG (sc-2027; Santa Cruz Biotechnology) did not show any staining. All sections were mounted with HardSet Mounting Medium with DAPI (Vector Labs), and photographed using a Nikon 80i Upright microscope using MetaMorph Software.

4.7. Quantification of Ki67 staining

The number of proliferating cells, stained by Ki67 antibody, was counted in six defined (140 × 140 μm) regions within the jaw mesenchyme of the mandibular sections of E16.5 embryos using ImageJ program. Three central-cut sections at least 22.5 μm apart were analyzed per bone.

4.8. TUNEL staining

Apoptotic cells were detected on frozen sections of heads by using In Situ Cell Death Detection Kit, TMR Red (Roche) according to the manufacturer's instructions.

4.9. Mandibular explant culture

Mandibles of E15.5 wild-type (WT) embryos were dissected in Hanks Balanced Salt Solution (HBSS, Sigma-Aldrich) and cultured in osteogenic medium containing β-glycerophosphate (8 mM; Sigma-Aldrich) and L-ascorbic acid phosphate (50 μg/ml; Wako), or in osteogenic medium supplemented with 10 ng/ml of VEGF164. Medium was replaced twice during the culture period. The mandibles were harvested on day 7 and subjected to Alizarin Red and Alcian Blue staining. Mandibular ossification was analyzed and quantified as described above. The entire experiment was repeated once and consisted of a total of 10 control and 10 VEGF164 treated mandibles.

Mandibles of E15.5 *Vegfa*^{fl/fl} embryos were dissected in HBSS and infected with either Ad-Cre or Ad-GFP (Vector Biolabs) for 24 h followed by culture in osteogenic medium. Protein was harvested from mandibles after 4 day culture for VEGF ELISA and Western Blotting analysis. Additional mandibles were infected with Ad-Cre and cultured in osteogenic medium (n = 3) or in osteogenic medium supplemented with 10 ng/ml of VEGF164 (n = 3).

After 7 days of culture, the mandibles were subjected to Alizarin Red and Alcian Blue staining, and mandibular ossification was analyzed.

4.10. ELISA assays

VEGF protein levels in mandibular lysates were assessed using the Quantikine Mouse VEGF Immunoassay (R&D Systems) in accordance with manufacturer's instructions.

4.11. Western blotting

Protein lysates of mandibles from mandibular explant culture were prepared and subjected to Western Blotting as described [21]. Primary antibodies used include: GFP (1:1000; ab290; Abcam), Collagen I (1:1000; ab21286; Abcam), Ihh (1:1000; ab39634; Abcam), β -catenin (1:1000; 9587; Cell Signaling), P-Smad1/5 (1:1000; 9516; Cell Signaling), HSP90 (1:1000; sc-13,119; Santa Cruz Biotechnology), and β -actin (1:5000; A5441; Sigma-Aldrich).

4.12. Statistical analysis

Results were presented as mean $\pm$ s.d. and unpaired student's t tests were used. P values <0.05 were considered significant.

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Abbreviations used

NCC	neural crest cell
Osx	Osterix
Col2	collagen II
Col1	collagen I

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Fig. 1. *Osx*⁺ and *Col2*⁺ progenitor cell populations are located in developing cranial and mandibular regions. (A) The plane of section for which the images in A, B and C are shown is indicated with the dashed line. *In situ* hybridization on frontal head sections of *Vegfa^{+/+}; Osx-Cre:GFP* mice at E14.5 (top) and E16.5 (bottom) for *Osx* (red) and *Col2a1* (turquoise) (scale bars: 400 μ m). To the right are magnified views of delineated areas of calvaria and mandible in images on left showing *Osx* and *Col2a1* expression (scale bars: 200 μ m). c, cartilage primordium of orbito-sphenoid bone; mk, Meckel's cartilage. (B) Analysis of different cell populations located in the head sections of E14.5 and E16.5 mice. Left: head sections from E14.5 (top) and E16.5 (bottom) *Vegfa^{+/+}; Osx-Cre:GFP* mice indicating *Osx/GFP⁺* cells. Right: head sections from E14.5 (top) and E16.5 (bottom) *Vegfa^{+/+}; Tomato; Col2-Cre* mice showing cells derived from *Col2*⁺ osteochondroprogenitor cells (scale bar: 400 μ m). (C) *In*

situ hybridization on frontal head sections of E14.5 *Vegfa*^{+/+}; *Osx-Cre:GFP* (top) and *Vegfa*^{fl/fl}; *Osx-Cre:GFP* (bottom) mice for *Osx* (red) and *Vegfa* (turquoise) (scale bars: 200 μ m). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Osx⁺ osteoblast progenitor cell-derived VEGF regulates intramembranous ossification in developing craniofacial bones. (A) Whole-mount staining with Alizarin Red and Alcian Blue of skulls of P1 *Vegfa*^{+/+}; *Osx-Cre:GFP* (top) and *Vegfa*^{fl/fl}; *Osx-Cre:GFP* (bottom) mice. Sagittal suture width indicated with double headed arrow. p, parietal bone; f, frontal bone; md, mandible; mk, Meckel's cartilage; pal, palatal bone. n = 9/8. Right graphs: Quantification of relative mandibular ossification (n = 8/8), relative mandibular length (n = 9/8), and relative width of sagittal suture (n = 6/6). Values represent mean values $\pm$ s.d. P <

0.01 for comparison between genotypes. (B) Top: The plane of section for which the images in B are shown is indicated with the dashed line. Bottom: Histological analysis of frontal head section of P1 *Vegfa*^{+/+}; *Osx-Cre:GFP* mice by H&E staining (scale bar: 400 μ m). To the right are magnified views of delineated areas of calvaria and mandible in bottom image on left showing Von Kossa staining, anti-Col1 staining, *Osx/GFP*⁺ expression, and Trap staining of P1 *Vegfa*^{+/+}; *Osx-Cre:GFP* and *Vegfa*^{fl/fl}; *Osx-Cre:GFP* mice (scale bar: 200 μ m). c, cartilage primordium of orbito-sphenoid bone; tb, tooth bud; mk, Meckel's cartilage.

Fig. 3.

VEGF derived from $Col2^{+}$ cells does not contribute to mineralization in developing craniofacial bones. (A) Whole-mount staining with Alizarin Red and Alcian Blue of skulls of P1 *Vegfa^{+/+}; Col2-Cre* (top) and *Vegfa^{fl/fl}; Col2-Cre* (bottom) mice. Sagittal suture width indicated with double headed arrow. p, parietal bone; f, frontal bone; md, mandible; mk, Meckel's cartilage; pal, palatal bone. $n = 9/9$. Right graphs: quantification of relative mandibular ossification ($n = 5/8$) and relative mandibular length ($n = 5/8$). Values represent mean values $\pm$ s.d. (B) Von Kossa staining of frontal head sections of calvaria and mandibles of P1 *Vegfa^{+/+}; Col2-Cre* (top) and *Vegfa^{fl/fl}; Col2-Cre* (bottom) mice (scale bar: 200 μ m). c, cartilage primordium of orbito-sphenoid bone; tb, tooth bud; mk, Meckel's cartilage.

Fig. 4.

Reduced initial ossification and thickness of the mandibular template upon loss of VEGF in *Osx*⁺ precursors. (A) Whole-mount staining with Alizarin Red and Alcian Blue of skulls of E15.5 *Vegfa*^{+/+}; *Osx-Cre:GFP* (top) and *Vegfa*^{fl/fl}; *Osx-Cre:GFP* (bottom) mice. md, mandible; mk, Meckel's cartilage. To the right are magnified views of delineated areas of mandible in images on left. (B) Left: *In situ* hybridization on frontal head sections of *Vegfa*^{+/+}; *Osx-Cre:GFP* (top) and *Vegfa*^{fl/fl}; *Osx-Cre:GFP* (bottom) mice at E16.5 for *Osx* (red) and *Col2a1* (turquoise) (scale bars: 200 μm). To the right are magnified views of delineated areas of mandible in images on left showing *Osx* and *Col2a1* expression (scale bars: 50 μm). Middle: mandibular sections from E16.5 *Vegfa*^{+/+}; *Osx-Cre:GFP* (top) and *Vegfa*^{fl/fl}; *Osx-Cre:GFP* (bottom) mice indicating *Osx/GFP*⁺ cells (scale bars: 200 μm). Mandibular thickness indicated with double headed arrow. Right: Anti-*Col1* staining of mandibular sections from E16.5 *Vegfa*^{+/+}; *Osx-Cre:GFP* (top) and *Vegfa*^{fl/fl}; *Osx-Cre:GFP* (bottom) mice (scale bars: 200 μm). tb, tooth bud; mk, Meckel's cartilage. Right graph: quantification of relative mandibular thickness. Values represent mean values ± s.d. *P* < 0.01 for comparison between genotypes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

Loss of VEGF in *Osx*⁺ precursors leads to increased cell proliferation without affecting vascular development within jaw mesenchyme. (A) TUNEL staining of mandibular sections from E16.5 *Vegfa*^{+/+}; *Osx-Cre:GFP* (top) and *Vegfa*^{fl/fl}; *Osx-Cre:GFP* (bottom) mice (scale bars: 200 μ m). (B) Anti-Ki67 staining of mandibular sections from E16.5 *Vegfa*^{+/+}; *Osx-Cre:GFP* (top) and *Vegfa*^{fl/fl}; *Osx-Cre:GFP* (bottom) mice indicating proliferating cells in tooth bud (left) and jaw mesenchyme (middle) (scale bars: 200 μ m). To the right are magnified views of delineated areas of jaw mesenchyme in images on left showing proliferating cells (scale bars: 50 μ m). (C) Left: Anti-CD31 staining of mandibular section from E16.5 *Vegfa*^{+/+}; *Osx-Cre:GFP* mouse showing *Osx/GFP*⁺ cells (scale bar: 200 μ m). tb, tooth bud; v, mandibular artery; mk, Meckel's cartilage. Right: magnified views of delineated area of mandible in image on left showing mandibular artery of E16.5 *Vegfa*^{+/+}; *Osx-Cre:GFP* (top) and *Vegfa*^{fl/fl}; *Osx-Cre:GFP* (bottom) mice (scale bar: 200 μ m). Arrows indicate CD31 expression in the mandibular artery and blood vessels, asterisk marks blood cells.

Fig. 6. Mandibular ossification is not stimulated by paracrine VEGF signaling and loss of VEGFR2 in *Osx*⁺ precursors does not affect intramembranous bone formation. (A) a. Overview of mandibular dissection procedure on E15.5 embryos. b. Schematic overview of the experimental procedures of the mandibular explant culture. c. Quantification of relative mandibular ossification (n = 5/5). Values represent mean values ± s.d. (B) *In situ* hybridization on frontal head sections of *Vegfr2*^{+/+}; *Osx-Cre:GFP* mice at E14.5 (top) and E16.5 (bottom) for *Osx* (red) and *Vegfr2* (turquoise) (scale bars: 400 μm). To the right are magnified views of delineated areas of mandible in images on left showing *Osx* and *Vegfr2* expression (scale bars: 200 μm). mk, Meckel's cartilage. (C) Whole-mount Alizarin Red and Alcian Blue-stained skeletal preparations of P1 *Vegfr2*^{+/+}; *Osx-Cre:GFP* (top) and *Vegfr2*^{fl/fl}; *Osx-Cre:GFP* (bottom) mice. md, mandible; mk, Meckel's cartilage. (For

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Fig. 7.

Reduced VEGF expression coincides with increased BMP signaling within jaw mesenchyme. (A) a. Schematic overview of the experimental procedures of the mandibular explant culture upon knockdown of VEGF. b. Western blotting of GFP and Col1 in whole mandibular lysates from *Vegfa^{fl/fl}* embryos treated with adenoviral Cre compared to adenoviral GFP (control). β -actin is loading control. c. Quantification of relative mandibular ossification ($n = 3/3$). Values represent mean values $\pm$ s.d. d. Western blotting of Ihh, β -catenin, and P-Smad1/5 in whole mandibular lysates from *Vegfa^{fl/fl}* embryos treated with adenoviral Cre compared to adenoviral GFP (control). HSP90 and β -actin are loading control. (B) *In situ* hybridization on frontal head sections of *Vegfa^{+/+}; Osx-Cre:GFP* (top) and *Vegfa^{fl/fl}; Osx-Cre:GFP* (bottom) mice at E14.5 for *Osx* (red) and *Bmp2* (turquoise) (scale bars: 200 μ m). To the right are magnified views of delineated areas of jaw

mesenchyme in images on left showing *Osx* and *Bmp2* expression (scale bars: 100 μ m). mk, Meckel's cartilage. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Fig. 8.

Model summarizing the paracrine and autocrine actions of VEGF in the jaw mesenchyme. VEGF produced by Osx^+ osteoblast progenitors stimulates mandibular ossification in an autocrine manner. In contrast, NCC-derived VEGF enhances vascular development *via* paracrine signaling, thereby regulating jaw extension and osteogenesis.