Neural crest cell-derived VEGF promotes embryonic jaw extension

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Jaw morphogenesis depends on the growth of Meckel's cartilage during embryogenesis. However, the cell types and signals that promote chondrocyte proliferation for Meckel's cartilage growth are poorly defined. Here we show that neural crest cells (NCCs) and their derivatives provide an essential source of the vascular endothelial growth factor (VEGF) to enhance jaw vascularization and stabilize the major mandibular artery. We further show in two independent mouse models that blood vessels promote Meckel's cartilage extension. Coculture experiments of arterial tissue with NCCs or chondrocytes demonstrated that NCC-derived VEGF promotes blood vessel growth and that blood vessels secrete factors to instruct chondrocyte proliferation. Computed tomography and X-ray scans of patients with hemifacial microsomia also showed that jaw hypoplasia correlates with mandibular artery dysgenesis. We conclude that cranial NCCs and their derivatives provide an essential source of VEGF to support blood vessel growth in the developing jaw, which in turn is essential for normal chondrocyte proliferation, and therefore jaw extension.

neural crest | VEGF | mandible | chondrocyte | blood vessel

Craniofacial abnormalities such as hemifacial microsomia, Treacher Collins syndrome, DiGeorge syndrome, and Goldenhar syndrome arise from developmental deficiencies in the first pharyngeal arch and are commonly associated with mandibular hypoplasia. These disorders are widely believed to result from a combination of inadequate formation, migration, or differentiation of cranial neural crest cells (NCCs). After delamination from the neural tube, cranial NCCs migrate into the mandibular primordia, where they interact with resident epithelium and mesoderm to initiate differentiation programs enabling formation of the skeletal components of the jaw (1). Cranial NCCs entering the mandibular primordia differentiate into prechondrocytes to form Meckel's cartilage, which then provides a scaffold for differentiating mandible bone. The proliferation and expansion of chondrocytes within Meckel's cartilage is also the major driving force behind jaw outgrowth before mandible bone formation (2). Although the signaling processes controlling prechondrocyte differentiation are well-established (3, 4), it is unclear which tissue types and signals instruct chondrocyte proliferation and morphogenesis of Meckel's cartilage.

VEGF (also known as VEGFA) is a secreted signaling protein that is made in three major isoforms termed VEGF121, VEGF165, and VEGF189 in humans and VEGF120, VEGF164, and VEGF188 in mice (5). Notably, mice lacking VEGF164 have craniofacial defects that include cleft palate, unfused cranial sutures, and shorter jaws (6). However, the source and precise role of VEGF in causing these defects have not yet been established. VEGF is best known for its pleiotropic roles in vascular development. In addition to supplying nutrients and oxygen for organ growth and tissue homeostasis, growing blood vessels also have the capacity to direct tissue morphogenesis. For example, blood vessels instruct pancreas and liver organogenesis as well as liver regeneration, and they promote osteogenesis and osteoblast differentiation during endochondral and intramembranous ossification (7–12). VEGF also has direct effects on nonendothelial cell types such as osteoblasts and osteoclasts and has been suggested to contribute to chondrocyte survival during long bone formation (13). Whether VEGF promotes craniofacial development via roles in blood vessel formation or bone or cartilage formation therefore remains to be established. Moreover, it needs to be investigated whether there is a link between VEGF and cranial NCC development during the formation of the skeletal structures of the face.

Here we show that cranial NCCs and their derivatives provide an essential source of VEGF to enable vessel growth and arterial stabilization in the embryonic mandible, and that loss of NCC-derived VEGF severely impairs chondrocyte proliferation in Meckel's cartilage and jaw outgrowth. We further show that in addition to their general role in supplying oxygen and nutrients, blood vessels secrete soluble factors that promote chondrocyte proliferation, explaining the key role of NCC-derived VEGF in directing mandible growth. Taken together with our finding that patients with hemifacial microsomia have mandibular artery dysgenesis that correlates with mandibular hypoplasia, we conclude that vascular development and jaw morphogenesis are intimately linked. Our study therefore provides direct insight into the mechanisms that regulate jaw development and the etiology of mandibular hypoplasia.

Significance

Craniofacial development is a complex morphogenic event that relies on highly orchestrated interactions between multiple cell types. Since the first description of Meckel's cartilage in the lower jaw more than 180 years ago, we have come to realize that expansion of this specialized structure underpins correct mandible development. Here we demonstrate that an intricate association between neural crest cells and blood vessels plays an important role in promoting chondrocyte proliferation and expansion of Meckel's cartilage as a prerequisite of correct mandibular morphogenesis. These findings provide direct insight into the origins and potential treatments of highly prevalent disorders affecting the mandible.

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Results

Mice Lacking VEGF in NCC-Derived Tissue Have Mandibular Hypoplasia. To investigate the mechanisms and cell types through which VEGF controls craniofacial development, we removed VEGF specifically in NCCs and NCC-derived tissue by crossing a floxed *Vegfa* allele with a *Wnt1-Cre* driver. *Wnt1-Cre;Vegfa*$^{fl/fl}$ mutants were born in the predicted Mendelian ratio but did not survive beyond the day of birth (Table S1). Compared with control *Vegfa*$^{+/fl}$ littermates, embryonic day (E) 17.5 mutants exhibited mild general bone hypoplasia of the cranium, including cleft palate and reduced ossification of the premaxillary and frontal bone (Fig. 1*A*). Strikingly, mutants displayed severe mandibular hypoplasia, with a disproportionately smaller mandible and Meckel's cartilage relative to the overall size of the skull. Moreover, the mandible was shorter and misshapen, with a bow-shaped rather than the extended arrowhead-shaped morphology seen in wild-type littermates (Fig. 1*A*). These findings show that loss of NCC-derived VEGF impairs mandible growth during development and raise the hypothesis that VEGF may have a direct effect on early NCC development. Prior chick studies have suggested that VEGF is expressed by surface ectoderm to promote migration of NCCs into the facial primordia (14); hence, NCC-derived VEGF may also be required for NCC migration. Staining of control and mutant *Wnt1-Cre;Vegfa*$^{fl/fl}$ embryos at E8.5 and E9.5 for NCC markers, including in situ hybridization for *Sox10* and immunostaining for SOX9 and p75, did not identify obvious defects in either NCC specification or immigration into the primordia of the mandible, also known as pharyngeal arch 1 (pa1) (Fig. S1*A*). In further support of this, lineage tracing of NCCs in *Wnt1-Cre;Vegfa*$^{fl/fl}$ mutants using a *R26R-YFP* reporter demonstrated that at E9.5 and E10.5, there was similar NCC density in pa1 (Fig. S1*B*). In situ hybridization for *Msx1* and *Dlx5* (Fig. S1*C*), which are downstream targets of the BMP-4 and Endothelin-1/dHAND signaling cascade (1, 15, 16), indicates patterning and specification of pa1 was normal in *Wnt1-Cre;Vegfa*$^{fl/fl}$ mutants. TUNEL and cleaved-Caspase-3 staining at E9.5 and E10.5 also showed no increase in cell death in *Wnt1-Cre;Vegfa*$^{fl/fl}$ mutants (Fig. S2). Together, these observations suggest that VEGF is not required cell-autonomously for early NCC development or survival. Therefore, the defects responsible for mandibular hypoplasia in mice lacking NCC-derived VEGF likely occur at a developmental point after NCC specification and migration into pa1.

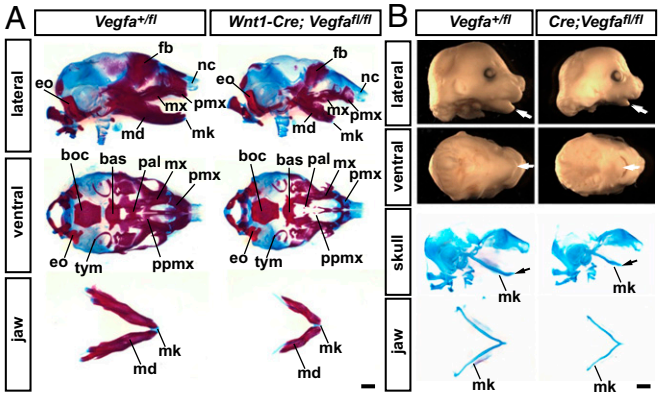

Fig. 1. Mice lacking VEGF in NCC-derived tissue have mandibular hypoplasia. (*A*) *Vegfa*$^{+/fl}$ (control) and *Wnt1-Cre;Vegfa*$^{fl/fl}$ (mutant) E17.5 skulls stained with Alizarin red and Alcian blue. bas, basisphenoid; boc, basioccipital; eo, exoccipital; fb, frontal bone; md, mandible; mk, Meckel's cartilage; mx, maxillary; nc, nasal capsule; pal, palatal; pmx, premaxillary; ppmx, palatal process maxillary; tym, tympanic. $n = 7/7$. (*B*, *Upper*) Mandibular hypoplasia is evident in mutant embryos at E14.5 (arrow). (*Lower*) Meckel's cartilage has not extended in mutants (arrow) and is misshapen. $n = 5/5$. (Scale bar, 400 μm.)

Loss of VEGF from the NCC Lineage Impairs the Normal Morphogenic Transformation of Meckel's Cartilage that Underlies Jaw Growth. To understand the origin of jaw defects in E17.5 *Wnt1-Cre;Vegfa*$^{fl/fl}$ mutants, we next performed a detailed analysis of cartilage and bone formation in the developing jaw of wild-type embryos. The analysis of cartilage formation revealed that the jaw normally undergoes a dramatic expansion and shape transformation between E13.5 and E14.5 (Fig. S3). Specifically, Meckel's cartilage transformed from an immature bow-shaped morphology, with both cartilage arms convex relative to each other, to a mature arrowhead-type morphology, with the cartilage arms concave relative to each other. In this mature form, the distal cartilage tips had flipped from a caudal to a rostral orientation. During this shape change, the cartilage also doubled in length. At E14.5, mandible ossification begins and Meckel's cartilage maintains its mature transformed shape for the remainder of gestation (Fig. S3). This growth and morphology transformation marks a previously undescribed developmental milestone in mandibular development that is likely a key step in ensuring the correct growth, shape, and function of the mature jaw.

We next asked whether *Wnt1-Cre;Vegfa*$^{fl/fl}$ mutants had failed to undergo this shape transformation of the mandible between E13.5 and E14.5. We observed that the heads of *Wnt1-Cre;Vegfa*$^{fl/fl}$ mutants at E14.5 were generally smaller than those of their control littermates, with a disproportionately shorter mandible (Fig. 1*B*). Ventral views of the jaw showed that the mandible had extended toward the maxillary extremity in control embryos, thereby concealing the tongue and oral cavity; however, the tongue and oral cavity were clearly visible in mutants because of the shorter jaw (arrows, Fig. 1*B*). Alcian blue staining of E14.5 heads revealed dysmorphology of Meckel's cartilage in mutants, with a failure of the cartilage arms to transform from a bowed to an arrowhead morphology, and this was accompanied by a failure of the distal cartilage tips to flip from a caudal to a rostral orientation (Fig. 1*B*, arrows in the lower panels). The persistence of this abnormal mandible morphology from E14.5 to E17.5 (compare Fig. 1*A* with Fig. 1*B*) in embryos that otherwise continued to develop to birth excluded the possibility of a general developmental delay as the cause of defective jaw development in *Wnt1-Cre;Vegfa*$^{fl/fl}$ mutants.

Neural Crest Cells Are the Major Source of VEGF in the Developing Jaw and Are Intimately Associated with Blood Vessels. To understand the role of NCC-derived VEGF in mandible development, we performed *Vegfa* in situ hybridization on wild-type and *Wnt1-Cre;Z/EG* reporter mice, which lineage trace all NCCs and their derivatives, as well as X-gal staining of *Vegfa*^{LacZ} reporter mice (17). At E9.5, *Vegfa* was strongly expressed by all NCCs and NCC-derived mesenchyme of pa1, as shown by overlapping expression of *Vegfa* mRNA and GFP in *Wnt1-Cre;Z/EG* reporter mice (Fig. S4*B*). GFP-positive NCCs began expressing *Vegfa* immediately on delamination from the neural tube and maintained high expression while migrating distally into pa1. This NCC-derived mesenchyme in pa1 was in close association with developing vasculature. *Vegfa* was also expressed by the neural tube and epithelial cells of pa1, which are not NCC-derived (GFP negative) (Fig. S4*B*, *Inset*). Expression of *Vegfa* was similar at E10.5, but was enriched in the anterior domain of pa1 (Fig. S4*C*, arrowheads).

Whole-mount immunostaining of the jaw at E11.5 identified many vessels in close association with SOX9-positive primordia of Meckel's cartilage, including a major blood vessel running lateral to Meckel's cartilage (Fig. S5). At E12.5, when the two cartilage arms had fused, this major blood vessel extended along the entire length of Meckel's cartilage into the distal jaw tip (Fig. S5). At this age, *Vegfa* was broadly and prominently expressed in the NCC-derived mesenchyme surrounding the condensing Meckel's cartilage compared with weaker expression in the cartilage itself (Fig. S6 *B* and *C*). Consistent with this, immunostaining at E12.5 showed that Meckel's cartilage was avascular, but surrounded by

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many small vessels (Fig. S6 *B* and *C*). The mandibular artery coursing along Meckel's cartilage was surrounded by NCC-derived cells and associated with an NCC-derived axon bundle (Fig. S6*C*).

At E14.5 and E15.5, expression of *Vegfa* remained lower in Meckel's cartilage compared with the surrounding mesenchyme (Figs. S7 and S8*B*). *Vegfa* expression was particularly enriched in the NCC-derived mesenchyme surrounding the distal tip of Meckel's cartilage, and this tissue was highly vascularized (Fig. S8*B*). Strikingly, *Vegfa* expression was enriched in the smooth muscle coating of the mandibular artery running lateral to Meckel's cartilage, which is exclusively of NCC origin (GFP-positive) (Fig. S8 *B* and *C*).

Vegfa in situ hybridization was also performed on *Wnt1-Cre; Vegfa*$^{fl/fl}$ mutant embryos, which revealed a lack of *Vegfa* expression in the NCC-derived mesenchyme of pa1 at E10.5 and in the mesenchyme surrounding Meckel's cartilage at E15.5 (Fig. S9), confirming NCCs as the major source of VEGF in the developing jaw. Thus, *Vegfa* expression in jaw mesenchyme and arterial smooth muscle supports the idea that NCC-derived VEGF functions in a paracrine fashion to promote vascular development, rather than acting cell-autonomously to promote the growth of chondrocytes in Meckel's cartilage. Together, these observations show that cranial NCC development is intimately associated with vascular development and that developing blood vessels are closely associated with Meckel's cartilage during jaw morphogenesis. Subsequent ossification of the mandible bone around Meckel's cartilage and its associated neurovascular bundle (Fig. S8*C*) leads to the formation of the mandibular and mental foramen through which the mandibular artery and nerve enter and exit the mature mandible bone (18).

Vascular Defects in *Wnt1-Cre;Vegfa*$^{fl/fl}$ Mutants Precede Mandibular Hypoplasia. Because VEGF promotes blood vessel growth, we next analyzed vascular development in the *Wnt1-Cre;Vegfa*$^{fl/fl}$ mandible before the bow-to-arrowhead transformation. Whole-mount immunostaining of control and *Wnt1-Cre;Vegfa*$^{fl/fl}$ mutant embryos with the vascular marker endomucin identified an obvious reduction in pa1 vascularization in mutants at E9.5 and E10.5 (Fig. 2*A*, arrows). Transverse sections through pa1 followed by quantitation of the endomucin-positive area confirmed a significant decrease in vascular density by almost 50% compared with controls (Fig. 2*B*). Costaining with the vascular endothelial marker CD31 and the vascular basement membrane marker collagen IV did not identify any basement membrane sleeves lacking endothelial cells in the mutant (Fig. S10). This suggests the decreased vascular density is a result of reduced angiogenesis, and not vessel regression. These findings demonstrate that VEGF secreted by NCC-derived mesenchyme is essential to promote normal vessel growth in the developing mandibular arch.

At E12.5, the gross morphology of the mandible was similar in mutants and controls (Fig. S11). SOX9 is a master regulator of chondrogenesis and is expressed in all chondroprogenitors and differentiated chondrocytes, where it directly promotes expression of *Col2a1* (collagen type II) (19, 20). Immunolabeling for SOX9, Alcian blue staining, and in situ hybridization for *Col2a1* showed similar specification and differentiation of chondrocytes within Meckel's cartilage in control and mutant embryos (Fig. 2*C* and Figs. S11 and S12). In contrast, immunostaining with CD31 showed that the microvessel density was decreased in the mutant relative to the control mandible (Fig. 2*C*). Moreover, the CD31-positive mandibular artery appeared significantly narrower in mutants compared with in wild-types (Fig. 2*C*). Loss of the mandibular artery along the length of Meckel's cartilage was also evident in

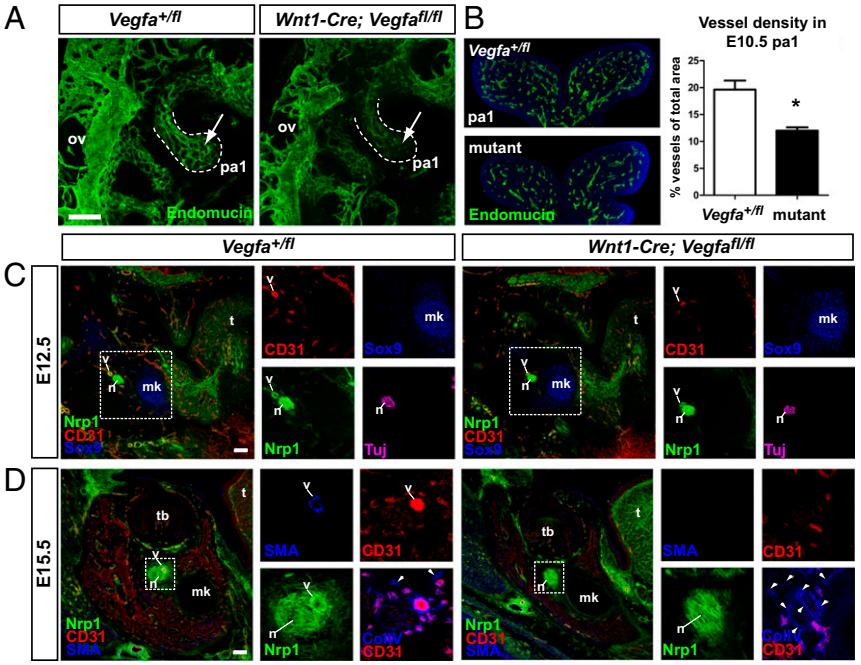

Fig. 2. Loss of VEGF from NCC-derived tissue reduces vessel density in the mandibular arch and causes regression of the mandibular artery. (*A*) Whole-mount imaging of vasculature immunostained with endomucin at E9.5 revealed an aberrant vascular network in pa1 (arrow); ov, otic vesicle. $n = 3/3$. (*B*) Frontal sections identified significantly reduced vascular density in E10.5 mutant embryos, $^*P < 0.05$; $n = 4/4$. (*C*) Immunostaining of frontal sections at E12.5 with CD31, SOX9, and NRP1 and a serial section with Tuj revealed normal specification of Meckel's cartilage in mutants. NRP1 and Tuj staining showed normal development of the mandibular nerve. The mandibular vessel (v) was substantially narrower and the microvascular density was reduced in mutants. $n = 3/3$. (*D*) Immunostaining of frontal sections through the mandible at E15.5 with CD31, SMA, and NRP1, and a serial section with CD31 and collagen IV. The mandibular artery was absent in mutant embryos, whereas the mandibular nerve remained intact. Collagen IV staining in the absence of CD31 staining indicates vessel regression in mutants (arrowheads). $n = 3/3$. (Scale bars: *A*, 200 μm; *C* and *D*, 100 μm.)

whole-mount staining of mutant jaws at E12.5 (Fig. S11). In contrast, the mandibular nerve [Tuj (betaIII-tubulin)- and NRP1 (neuropilin 1)-positive] appeared similar in the neurovascular bundle of mutants and wild-types (Fig. 2*C* and Fig. S11).

At E15.5, when mandibular hypoplasia was macroscopically obvious in mutants, the mandibular artery had atrophied, as indicated by loss of both CD31 and smooth muscle actin (SMA) staining in the area adjacent to the mandibular nerve (Fig. 2*D*). Costaining for CD31 and collagen IV identified many basement membrane sleeves lacking endothelial cells in the mutant, but not control mandible, suggesting vessel regression (Fig. 2*D*, arrowheads). In contrast, vessels coated primarily by non-NCC-derived smooth muscle were unaffected in *Wnt1-Cre;Vegfa*$^{fl/fl}$ embryos (Fig. S13). Together, these observations suggest a selective dependence of the mandibular artery on VEGF secreted from NCC-derived smooth muscle. In summary, loss of NCC-derived VEGF impairs jaw vascularization through reduced microvessel formation and by causing regression of the mandibular artery.

NCC-Derived VEGF Promotes Vessel Growth in Vitro. To provide further evidence that NCC-derived VEGF promotes vessel growth, primary embryonic NCCs and aortic rings from wild-type adult mice were cocultured in a collagen matrix in medium lacking VEGF. Under these culture conditions, primary NCCs or the addition of recombinant VEGF similarly promoted vessel sprouting from the aortic rings (Fig. 3*A* and Fig. S14). Addition of soluble VEGFR1 (sFLT1) to trap secreted VEGF (21) inhibited NCC-induced vessel sprouting (Fig. 3*A*), demonstrating that NCCs secrete VEGF to promote vessel growth. Together with the observation of reduced mandible vascularization in mutants lacking NCC-derived VEGF, these findings are consistent with a model in which NCCs populating pa1 secrete VEGF to ensure the appropriate vascularization of the developing mandibular arch.

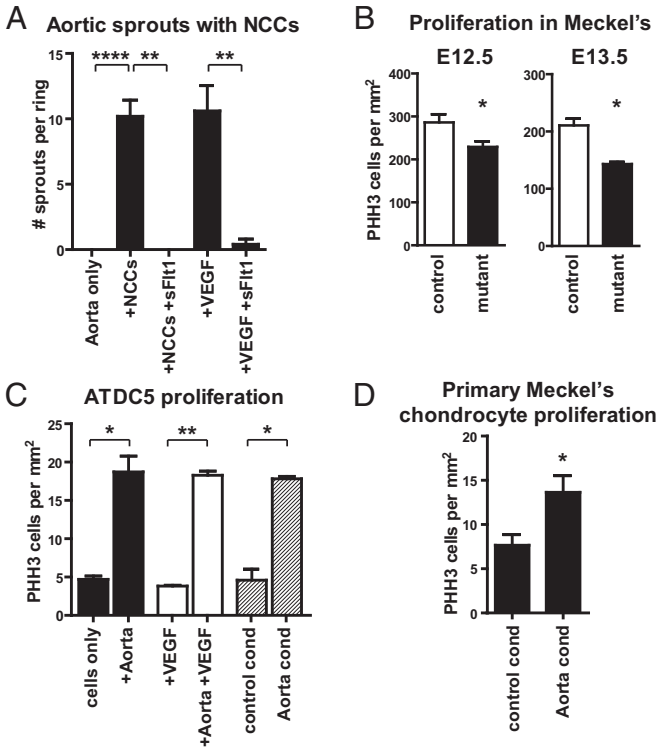

Fig. 3. NCC-derived VEGF induces vessel growth, and vessels induce chondrocyte proliferation. (*A*) Aortic rings sprout when cocultured with NCCs or when induced with recombinant human VEGF165. Quantitation of sprouting from five to 10 aortic rings in two independent experiments. ****$P <$ 0.0001; **$P < 0.01$. (*B*) Chondrocyte proliferation in Meckel's cartilage at E12.5 and E13.5. Data are mean of $n = 3$ embryos; E12.5, *$P = 0.0109$; E13.5, *$P = 0.0195$. (*C*) Proliferation of ATDC5 cells cocultured with aortic rings, with or without VEGF, or treated with aorta conditioned media. Data are mean of $n = 3$ experiments. *$P < 0.05$; **$P < 0.01$. (*D*) Proliferation of primary Meckel's cartilage chondrocytes when treated with aorta conditioned media. Data are mean of $n = 3$ experiments. *$P = 0.0178$.

Blood Vessels Promote Chondrocyte Proliferation. The findings described here suggest that NCC-derived VEGF and VEGF-induced blood vessels are major factors in the transformation of Meckel's cartilage that promotes jaw expansion and elongation. We therefore examined whether reduced mandible growth in *Wnt1-Cre;Vegfa*$^{fl/fl}$ mutants is preceded by defective cell proliferation or survival in Meckel's cartilage preceding the mandible defect becoming macroscopically obvious. Staining for the mitosis marker phospho-histone H3 (PHH3) showed that proliferation of chondrocytes was significantly reduced in mutants by ∼20% and ∼30% at E12.5 and E13.5, respectively, whereas TUNEL staining did not show altered apoptosis (Fig. 3*B* and Fig. S15). This suggests that defective mandible growth is a result of reduced chondrocyte proliferation, and not excessive cell death.

Reduced cell proliferation in Meckel's cartilage may have been caused by the loss of VEGF acting directly on chondrocytes; in support of this hypothesis, VEGF has been shown to promote chondrocyte survival during epiphyseal cartilage formation (22, 23). Alternatively, loss of cell proliferation in Meckel's cartilage may be a result of the reduction in VEGF-induced blood vessels that normally promote chondrocyte proliferation. To distinguish whether VEGF promotes chondrocyte proliferation directly or indirectly via blood vessels, we cultured the chondrogenic cell line ATDC5 in the presence or absence of recombinant VEGF and/or aortic rings and assessed chondrocyte proliferation by immunostaining for PHH3. We observed that recombinant VEGF did not increase chondrocyte proliferation (compare panels labeled "cells only" and "+VEGF" in Fig. 3*C* and Fig. S16 for alternate VEGF isoforms). In contrast, aortic rings were found to induce chondrocyte proliferation (Fig. 3*C* and Fig. S17*A*). Moreover, conditioned media from cultured aortic rings was also effective in inducing chondrocyte proliferation (Fig. 3*C*). These findings agree with the expression pattern of *Vegfa* during mandible development, which shows prominent expression of VEGF in the vicinity of blood vessels, but not in Meckel's cartilage, and are consistent with a role for NCC-derived VEGF in jaw vascular development, rather than direct effects on chondrocytes in Meckel's cartilage. Together, these findings further support the idea that blood vessels, in addition to providing general trophic support, also secrete soluble factors that promote Meckel's cartilage proliferation. Indeed, conditioned media from aortic rings also promoted proliferation of cultured primary Meckel's cartilage chondrocytes (Fig. 3*D* and Fig. S17*B*).

Mandibular Artery Defects Cause Mandibular Hypoplasia in *Tie2-Cre; Nrp1*$^{fl/fl}$ Mice. To provide additional evidence that blood vessels promote jaw extension, we next examined *Tie2-Cre;Nrp1*$^{fl/fl}$ mutant mice because they have well-characterized vascular defects (24) and were reported to display craniofacial defects at low penetrance (25). NRP1 is an isoform-specific receptor for VEGF in neurons that also promotes VEGF-independent extracellular matrix signaling in endothelial cells to enable central nervous system angiogenesis (26–28). Moreover, VEGF binding to NRP1 promotes arterial development in the heart and smooth muscle cell coverage of retinal arteries (29, 30). *Tie2-Cre;Nrp1*$^{fl/fl}$ mutant mice therefore present a complementary model to *Wnt1-Cre;Vegfa*$^{fl/fl}$ mice to validate a role for blood vessels in jaw development.

CD31 immunolabeling of transverse sections through the E13.5 mandible revealed that *Tie2-Cre;Nrp1*$^{fl/fl}$ mutants did not exhibit an overall reduction in vascular density in the embryonic jaw

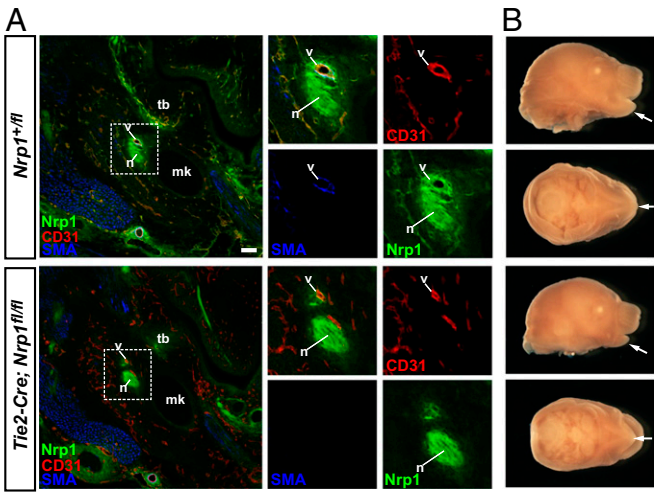

Fig. 4. Vessel defects correlate with mandibular hypoplasia in *Tie2-Cre; Nrp1*$^{fl/fl}$ mutants. (*A*) Immunostaining of frontal sections through the mandible with CD31, SMA, and NRP1 in E13.5 control and *Tie2-Cre;Nrp1*$^{fl/fl}$ mutant embryos. n, nerve; v, vessel. $n = 3/3$. (*B*) Mandibular hypoplasia in E15.5 *Tie2-Cre;Nrp1*$^{fl/fl}$ mutants and littermate controls. Note reduced extension of the mandible to the maxillar extremity (arrows). $n = 3/4$. (Scale bar, 100 μm.)

(Fig. 4*A*). However, the mandibular artery of *Tie2-Cre;Nrp1*$^{fl/fl}$ mutants at both E13.5 and E15.5 was significantly narrower and lacked a smooth muscle coating (Fig. 4*A* and Fig. S18). Correlating with impaired mandibular artery development, mandibular extension was reduced in three of four *Tie2-Cre;Nrp1*$^{fl/fl}$ mutants at E15.5 (Fig. 4*B*, arrows). This defect was milder than that of *Wnt1-Cre;Vegfa*$^{fl/fl}$ mutants, suggesting a defective mandibular artery impairs jaw extension, but also that general vascular deficiency, as observed in *Wnt1-Cre;Vegfa*$^{fl/fl}$ mice, exacerbates jaw defects. Our findings in two independent mouse models therefore indicate that defective vascular patterning in the jaw causes mandibular hypoplasia that can be explained by a role of blood vessels in promoting chondrocyte proliferation.

Mandibular Hypoplasia Is Linked with Loss of the Mandibular Artery in Patients with Hemifacial Microsomia. Our findings in mouse models show that aberrant vessel growth contributes to craniofacial jaw defects. In agreement, a prior study suggested that craniofacial defects correlated with hematoma of the stapedial artery in thalidomide-treated mouse embryos (31). Moreover, this previous study also proposed that embryonic vascular insults can give rise to hemifacial microsomia, a clinical syndrome of unclear etiology that variably affects the development of the facial skeleton and soft tissues including the ears, mouth, facial musculature, and mandible (32). We therefore examined computed tomography and X-ray images of the mandibles from individuals with severe hemifacial microsomia (clinical classification skeletal, auricle, and soft tissue score of 3; $n = 6$). Specifically, we assessed whether the mental foramen at the distal tip of the chin was present as it marks the exit point of the mandibular artery and nerve from the mandible bone. As the clinical phenotype is generally restricted to one side of the face in these patients, the unaffected side provided an internal reference point for the correlation between vessel and mandibular defects. In all patients examined, the mental foramen was absent on the affected side of the mandible, but present on the unaffected side (Fig. 5*A*). In addition, the mandibular foramen, through which the artery enters the mandible, was narrower and mispositioned on the affected side of the jaw (Fig. 5*B*). X-ray imaging further demonstrated that the canal of the artery and nerve extended through the bone from the mandibular to the mental foramen on the unaffected side, whereas this canal was narrower and did not extend the length of the mandible on the affected side (Fig. S19). Because the artery is established before the onset of ossification during embryogenesis (18), these observations suggest the mandibular artery was absent on the affected side during critical stages of jaw development in patients with hemifacial microsomia.

Discussion

Mandibular hypoplasia contributes to the pathogenesis and clinical pathology of many craniofacial disorders. Understanding the origins of these disorders and the mechanisms controlling mandible growth are essential to enable the design and implementation of innovative diagnostics and therapeutics. We have shown here that NCC-derived VEGF is essential for jaw vascularization to promote chondrocyte proliferation and subsequent elongation and shape transformation of Meckel's cartilage.

VEGF is well known for its pleiotropic roles in vessel formation, and it also promotes the survival and proliferation of various nonvascular cell types. For example, VEGF has previously been shown to promote chondrocyte survival before endochondral bone formation (23). In contrast, our study found no evidence in favor of paracrine or autocrine effects of VEGF on chondrocyte proliferation or survival in jaw development. Instead, our coculture experiments suggest that blood vessels, which require VEGF for their formation, secrete soluble factors that then promote chondrocyte proliferation. Consistent with the idea that blood vessels play an important role in cartilage morphogenesis, we observed that *Wnt1-Cre;Vegfa*$^{fl/fl}$ mutants have vascular growth and stabilization defects before notable jaw defects, even though the early formation, migration, and condensation of NCCs into SOX9-positive cartilage progenitors is unaffected. Moreover, *Tie2-Cre;Nrp1*$^{fl/fl}$ mutants with defective arterial stabilization also had shorter jaws. Taken together, our data therefore fit best with a model in which NCC-derived VEGF promotes vascular development in the jaw, with blood vessels being an essential source of trophic or secreted factors to stimulate chondrocyte proliferation in Meckel's cartilage, thereby enabling its growth and shape transformation (Fig. S20).

Even though NCCs are known to interact with the dorsal aorta (33) and pharyngeal arch arteries (34) to establish the sympathetic nervous system and promote remodeling of the cardiac outflow tract, a specific role for NCCs in stimulating angiogenesis or stabilizing arteries has not previously been described. Our findings that NCC-derived VEGF is essential for vessel growth, and that VEGF secreted from NCC-derivatives, such as smooth muscle, promotes arterial stabilization, therefore add to the growing list of codependencies between NCC and vascular development.

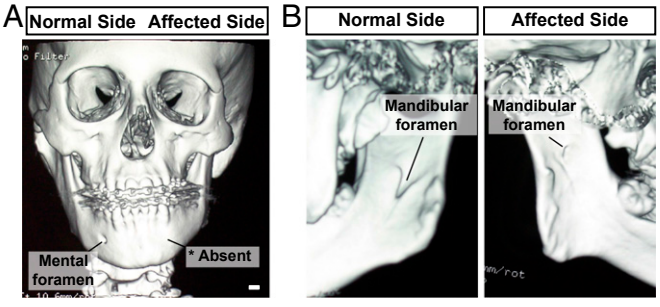

Fig. 5. Vessel defects correlate with mandibular hypoplasia in hemifacial microsomia. (*A*) Computed tomography image of a 15-y-old male with a hypoplastic mandible on his left (affected) side. The mental foramen is absent on the affected side of the jaw. (*B*) Computed tomography images indicate the mandibular foramen is present on the unaffected side, but is distinctly smaller in diameter and mispositioned on the affected side. (Scale bar, 1 cm.)

Although a primary role of blood vessels is to provide nutrients and oxygen to support tissue growth, vessels have also been shown to provide instructive signals for tissue-specific differentiation programs in the liver, pancreas, and sympathetic nervous system, and they also regulate liver regeneration and osteogenesis (7–10, 12, 33). Our in vitro findings suggest an additional angiocrine role for blood vessels as a source of secreted signals for cartilage proliferation. Such a role for blood vessels has not previously been documented for cartilage formation, neither during craniofacial morphogenesis nor elsewhere in the body. Our study therefore extends knowledge of the processes that enable skeletal development by identifying blood vessels, in addition to their role in promoting osteoblast differentiation (8, 11, 12), as important regulators of chondrocyte proliferation. Future studies should therefore aim at identifying the vessel-derived factors that promote cartilage growth to provide insight into the molecular mechanisms of cartilage growth and craniofacial development.

Our finding that defective blood vessel development leads to mandibular hypoplasia differs from prior studies in mice, which identified precocious NCC death (i.e., Treacher Collins syndrome) (35), abnormalities of pharyngeal arch growth (i.e., DiGeorge syndrome) (36), or aberrant cartilage specification (i.e., Pierre Robin Sequence) (3) as causes of craniofacial defects. Our model of developmental vascular deficiency as a cause of craniofacial birth defects was corroborated by studies in patients with hemifacial microsomia. Although we cannot exclude NCC defects in these patients, the lack of a mental foramen and vascular canal on the affected side of the jaw in this patient cohort suggests the mandibular artery was absent during the critical time of jaw extension when ossification occurred around Meckel's cartilage. This identifies vascular defects as an additional causative factor for craniofacial malformations, and in the future, it will therefore be important to investigate the prevalence of vascular defects in patients with hemifacial microsomia and other similar disorders.

We conclude that VEGF expression by NCCs ensures adequate vessel growth and arterial stability in the jaw, and that vessel-derived factors in the developing jaw enable appropriate levels of chondrocyte proliferation and morphogenesis of Meckel's cartilage as essential prerequisites for normal jaw extension. Our findings therefore provide insight into the etiology of craniofacial birth defects and open the field to further studies into the signaling molecules and/or trophic factors that are supplied by blood vessels to promote cartilage and craniofacial development.

Experimental Procedures

Animals. All experiments were carried out in accordance with ethical guidelines of the SA Pathology Animal Ethics Committee and UK Home Office. To remove VEGF specifically in NCCs, we crossed *Wnt1-Cre;Vegfa*$^{fl/+}$ males to *Vegfa*$^{fl/fl}$ females (37, 38). Lineage tracing was performed with Z/EG or R26R-YFP mice (39, 40). *Vegfa*^{Lacz} embryos (17) were used to visualize VEGF expression with β-galactosidase activity. To remove Nrp1 specifically in blood vessels, we crossed *Tie2-Cre* mice to *Nrp1*$^{fl/fl}$ mice (24). Additional methods are described in the *SI Experimental Procedures*.

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