ORIGINAL ARTICLE

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FGF signaling in mandibular skeletogenesis

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Structured Abstract

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Objective – To examine the functions of FGF/FGFR signaling during mandibular skeletogenesis *in ovo*.

Design – We examined the effects of inhibition of FGF signaling during mandibular skeletogenesis by overexpressing replication-competent RCAS virus encoding a truncated form of FGFR3 in the chicken mandibular process between stages 17 and 26.

Results – Injection of RCAS-dnFGFR3 into the developing mandible resulted in abnormalities in a stage- and region-dependent manner. Injection at early stages of development resulted in the truncation of Meckel's cartilage, severely reduced outgrowth of the mandibular process and absence of five of the mandibular bones. Injection at later stages did not affect the outgrowth of the mandibular process and Meckel's cartilage but resulted in abnormalities in mandibular osteogenesis in a region-specific manner. The bones in the more caudal region were frequently truncated whereas bones in the more rostral regions such as dentary and splenial bones were frequently absent.

Conclusion – Together these experiments have revealed essential roles for FGF/FGFR signaling in the elongation of Meckel's cartilage, development of osteogenic condensations and appositional growth of mandibular bones.

Key words: chick embryo; chondrogenesis; fibroblast growth factor receptor 3; mandibular outgrowth; Meckel's cartilage; osteogenesis

Introduction

The discoveries that mutations in fibroblast growth factor receptors (FGFRs) are the etiology of more than 15 human disorders involving cartilage and/or bone dysplasia defined essential roles for FGF signaling in skeletal development (1). Several of the disorders caused by mutations in FGFRs affect the cranial skeleton, the majority of which is derived from cranial neural crest (2).

fibroblast growth factor receptors belong to a family of highly conserved transmembrane tyrosine kinases that serve as high affinity receptors for FGFs. To date, four FGFRs and at least 22 FGF ligands have been identified (3). FGFRs normally exist as inactive monomers and upon ligand binding, become dimerized and activated. Activated FGFRs serve as binding sites for proteins with SH2 domains. The recruitment of SH2 domain proteins

results in phosphorylation and activation of downstream signaling intermediates and complex signal transduction cascades (1–3).

The expression of several members of the FGFs and FGFRs in the developing mandibular processes in many vertebrates suggests conserved roles for FGF signaling in mandibular morphogenesis (1, 4). The chick offers a favorable system to study the signaling networks regulating various aspects of mandibular morphogenesis, as it is accessible to experimental manipulation *in ovo*. In the chick embryo, the mandibular processes are recognizable at stage 15 (E2.5). Between stages 15 and 25 the mandibular process contains undifferentiated mesenchyme. Skeletogenesis of the developing mandible occurs in the following few days. The chondrogenic condensations form first in the caudal region at around stage 25/26 (E5). Later at around stage 28 (E6) cells within the chondrogenic condensations differentiate into chondrocytes and begin synthesis and secretion of cartilage-specific extracellular matrix proteins. Chondrogenic differentiation occurs first in the caudal region and later extends into the more rostral region. Further morphogenesis and elongation of Meckel's cartilage during embryogenesis is mediated by several mechanisms including appositional and interstitial growth. The elongation of Meckel's cartilage also involves addition of new chondrogenic cells to the tips of the growing ends of the chondrogenic condensations and newly formed cartilage.

The mandibular arch also contains six mandibular bones (angular, supra-angular, articular, splenial, dentary, and mentomandibular) that are formed by intramembranous ossification. The osteogenic condensations are formed first in the caudal region at around stage 31 (E7–7.5) and later in the more rostral regions. The first site of mineralization is seen around stage 35 (E9) in the articular, angular and supra-angular bones in the caudal region of the developing mandible (Fig. 1A). The caudal bones enlarge and fuse (Fig. 1B), followed by mineralization of the bones in more rostral regions (splenial, dentary, and mentomandibular) that occurs at around stage 37 (E11) (Fig. 1C). At stage 38 (E12) the mandibular processes contain fully differentiated Meckel's cartilage surrounded by six mandibular bones (Fig. 1D).

In situ hybridization studies showed the expression of *Fgfr3* at various stages of chondrogenesis and intramembranous ossification in the chick mandible (5) suggesting roles for FGFR3 in chondrogenesis and osteogenesis of neural crest derived mandibular mesenchyme.

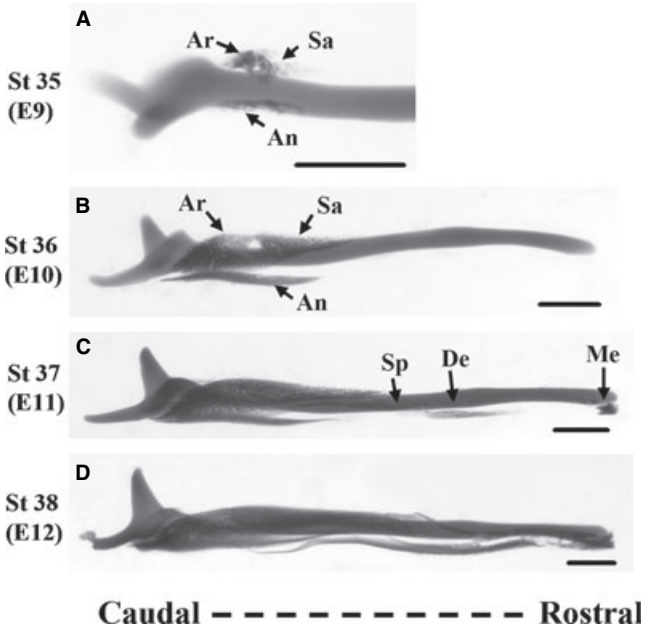


Fig. 1. Temporal development of chicken mandibular bones. (A–D) Lateral views of whole mount skeletal staining of the mandible between stages 35 and 38 (D9–D12). (A) The alizarin red staining shows the calcification of articular, supra-angular and angular bones in the caudal region of the chick mandible at stage 35. (B) The alizarin red staining at stage 36 shows the growth and fusion of the articular, supra-angular and angular bones. There is no alizarin red staining in the more rostral mandibular bones. (C) The alizarin red staining at stage 37 shows the calcification of the splenial, dentary and mentomandibular bones. (D) The alizarin red staining at stage 38 shows the outgrowth and fusion of all mandibular bones. Scale bars = 1 mm. Ar, articular; Sa, supra-angular; An, angular; Sp, splenial; De, dentary; Me, mentomandibular.

Here, we examined the effects of inhibition of FGFR3 signaling in mandibular skeletogenesis by overexpressing replication-competent RCAS virus encoding truncated forms of FGFR3 *in ovo*. We show that inhibition of FGFR3 signaling resulted in abnormalities in the developing Meckel's cartilage and five of the mandibular bones in a stage- and region-dependent manner.

Materials and methods

Production of retroviruses carrying a truncated form of FGFR3c

A viral construct containing a truncated form of murine FGFR3c spanning amino acids 1 through 416 with a carboxy-terminal six-myc-epitope tag was constructed

and used. Viral constructs containing no inserted construct (RCAS) or containing the coding sequence of green fluorescent protein (GFP) (RCAS-GFP) were used as controls (6).

Preparation of viral stocks and microinjections into developing chick mandible

Viral stocks of about 1×10^9 pfu/ml were prepared and purified as described (7, 8). Fertilized pathogen-free white leghorn chick eggs (SPAFAS; Charles River Laboratories, Franklin, CT, USA) were incubated at 37.5°C in a humidified incubator and embryos were staged according to Hamburger and Hamilton (9). Concentrated virus mixed with 4% (v/v) fast green was injected into the developing mandible of 92 stage 17–26 (E3–E5) embryos. After injection, eggs were sealed, and returned to the incubator for various times.

Skeletal whole-mount staining and analysis

Embryos were harvested at stages 37 (E11) and 40 (E14) and processed for staining with alcian blue and alizarin red as described (10). Stained heads were cleared, visualized and photographed under a dissecting microscope equipped with a SPOT RS digital camera (Sterling Heights, MA, USA). The length of Meckel's cartilage was analyzed in 30 embryos using ImageJ software (US National Institute of Health, available on the Internet from http://rsb.info.nih.gov/ij/download.html).

MicroCT analysis

Mandibles were collected from two embryos and fixed overnight in 70% ethanol at 4°C. Mandibles were scanned at 16 μm intervals with a Scanco Medical μCT 42 (Scanco Medical AG, Bassersdorf, Switzerland) with the following parameters; 45 kV, 177 micro-amps and 300 ms. Visualization and measurement of bone volume (mm^3) on the entire injected and uninjected sides were performed using semi-automated computer software.

Tissue fixation, processing, and analysis

Tissue fragments from four embryos were fixed in freshly prepared 4% paraformaldehyde in PBS at 4°C overnight, dehydrated and embedded in paraffin. Seven-micrometer sections were mounted on Probe-On Plus slides and processed for various histological analyses. Sections were stained with hematoxylin, eosin, and Alcian blue.

In situ hybridization

In situ hybridization using antisense ^{33}P-labled RNA probes was performed as described (11). The cDNAs for this study included *Fgfr3* (11), *Noggin* (12), *Sox9* (13), aggrecan core protein (14), *PTH-1R* (15), *Runx2* (16), and *Osteocalcin* (17). Individuals who kindly provided these cDNAs include Drs L. Niswander (*Noggin*), C. Healy (*Sox9*), M. Tanzer (aggrecan core protein), C. Tabin (*PTH-1R*), S. Sticker (*Runx2*), and L. Gerstenfeld (*Osteocalcin*).

Following *in situ* hybridization, the sections were stained with hematoxylin and mounted with permount. Using Adobe Photoshop 7.0 software (San Jose, CA, USA), the silver grains in the dark-field image were selected, colored red, and then superimposed onto the bright-field images.

Statistical analysis

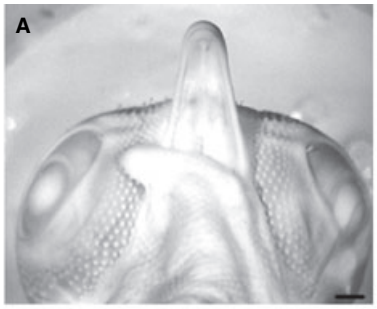
Unpaired, two-tailed *t*-tests were performed to determine statistically significant differences. All values were expressed as the mean ± SE, and $p < 0.01$ was considered statistically significant.

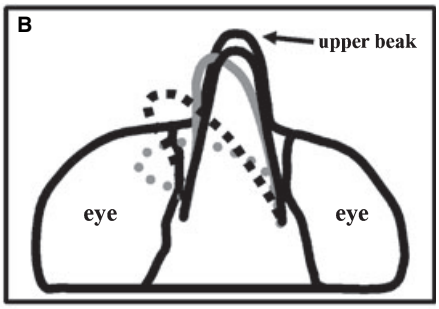
Results

To study the effects of blocking FGFR3 signaling on mandibular morphogenesis *in ovo*, RCAS-dnFGFR3 or control viruses were injected into the right half of the mandible of chick embryos between stages 17 and 26. Embryos were harvested 6–8 days after injection (stage 37, E11) and processed for skeletal staining.

Stage-dependent effects of RCAS-dnFGFR3 on mandibular morphogenesis

Embryos injected with RCAS-dnFGFR3 displayed stage-dependent deviation of the mandibular processes away from the upper beak on the injected side (Fig. 2). Deviation was observed in 95% (39/41) of embryos injected between stages 17 and 20, 100% (16/16) of embryos injected at stage 23/24 and 12% (3/25) of the embryos injected at stage 26 (Fig. 2C). The severity of





Normal (no deviation) Moderate deviation

Mild deviation Severe deviation

C

Deviation	Stage 17-20 (n=41)	Stage 23/24 (n=16)	Stage 26 (n=25)
Severe	73% (30)	38% (6)	0%
Moderate	12% (5)	31% (5)	0%
Mild	10% (4)	31% (5)	12% (3)
None	5% (2)	0%	88% (22)

Fig. 2. Effects of over-expression of dnFGFR3 on mandibular outgrowth. (A) Inferior view of a stage 37 chicken head injected with RCAS-dnFGFR3 at stage 17/18. Note the severe deviation of the mandible towards the injected side in reference to the upper beak. (B) Schematic line drawing representing the various degrees of deviation in mandibular processes on the injected side. The severities of the abnormalities in the mandibular processes are based on the distance and location of the tip of the lower beak from upper beaks shown in line drawing (B). (C) Percentage of various degrees of mandibular deviation in stage 37 embryos following injections of RCAS-dnFGFR3 at various stages of development.

deviation from the upper beak was also stage-dependent (Fig. 2C). Injection between stages 17 and 20 resulted in severe deviation of the mandible in a significantly high percentage of embryos, whereas injection at stage 26 led to only mild deviation in a small percentage of embryos. Injection at stage 23/24 led to similar percentages of severe, moderate, and mild deviations (Fig. 2C). All embryos injected with control RCAS virus did not display deviations of the mandibular processes, indicating that viral infection itself does not disturb mandibular outgrowth.

Stage-dependent effects of RCAS-dnFGFR3 on morphogenesis of Meckel's cartilage

Skeletal staining at stage 37 of the heads injected with RCAS-dnFGFR3 showed stage-dependent abnormalities in Meckel's cartilage and mandibular bones on the injected sides (Fig. 3). Injections of RCAS-dnFGFR3 at all stages did not appear to affect the development of the muscles in the tongue (data not shown). Skeletal development in embryos infected with control viruses was indistinguishable from uninfected embryos (Fig. 3A), indicating that virus infection itself does not affect mandibular skeletogenesis.

Injection at stage 23/24, led to truncations of Meckel's cartilage (17%, 2/12), abrupt bends (50%, 6/12), and discontinuities (25%, 3/12) in the middle third of Meckel's cartilage (Fig. 3C and data not shown). Injection at stage 26 did not affect the length of the Meckel's cartilage but resulted in variations in the thickness of Meckel's cartilage and gradual bends in Meckel's cartilage along the oral/aboral axis (50%, 3/6) (Fig. 3D). The most severe defects in the development of Meckel's cartilage were observed after injection of RCAS-dnFGFR3 between stages 17 and 20 (Fig. 3B). A high percentage (83%, 5/6) of injected embryos exhibited severe truncation/shortening of Meckel's cartilage (Fig. 3B). Morphometric analysis of mandibles in these embryos showed a close correlation between the severity of defects in mandibular outgrowth and the severity of the truncation in Meckel's cartilage in the treated sides (Table 1). At stage 37 (8 days after injection) the length of Meckel's cartilage in animals with severe defects in mandibular outgrowth was significantly shorter than those with moderate and mild deviations (Table 1).

These observations suggested that defects in mandibular outgrowth are related to the negative effects of dnFGFR3 on the growth of Meckel's cartilage. To better understand the underlying mechanisms leading to

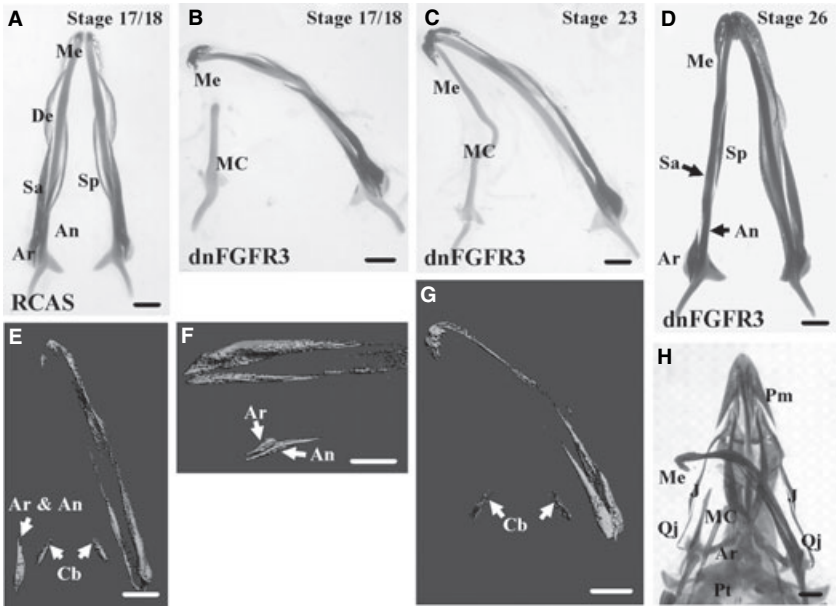


Fig. 3. Stage-specific effects of over-expression of dnFGFR3 on Meckel's cartilage and mandibular osteogenesis. Whole-mount skeletal preparation of mandibles after injection with control virus (A) or RCAS-dnFGFR3 (B, C, D) into the right mandibular process at stages 17/18 (A, B) stage 23 (C) and stage 26 (D). All embryos were harvested at stage 37. In all pictures, the injected side is on the left. (A) Normal skeletal structures in both sides in a mandible injected with control RCAS at stage 17/18. (B) Truncation of Meckel's cartilage, severe defect in outgrowth of the mandibular process, and absence of all bones except for the mentomandibular bone on the injected side in a mandible after injection of RCAS-dnFGFR3 at stage 17/18. (C) Bends in Meckel's cartilage, mild defect in outgrowth of the mandibular process, and absence of all bones except for the mentomandibular bone on the injected side in a mandible after injection of RCAS-dnFGFR3 at stage 23. (D) Injection of RCAS-dnFGFR3 at stage 26 led to truncations of the mentomandibular, splenial, angular, supra-angular, and articular bones. The dentary bone is absent. There are no apparent defects in the outgrowth of the mandibular process and Meckel's cartilage. (E–G) MicroCT images of stage 37 mandibles injected with RCAS-dnFGFR3 at stage 17/18. The absence of the dentary, splenial, and supra-angular bones and truncations of the angular, articular and mentomandibular bones are shown in the superior (E) and lateral (F) microCT images. Morphometric analysis demonstrated an 86% reduction in bone volume on the treated side compared with the untreated side. (G) An inferior microCT image of another mandible. Note the truncated mentomandibular bone and the absence of all other bones on the treated side. Morphometric analysis demonstrated a 100% reduction in bone volume on the treated side compared with the untreated side. Mineralization in the ceratobranchial hyoid cartilage, which undergoes endochondral ossification, is evident on the injected and uninjected side. (H) An inferior view of a stained head from an animal injected at stage 17/18 with RCAS-dnFGFR3 and isolated at stage 40 (11 days after injection). Note that the pattern of osteogenesis is similar to those in (B) indicating that injection of RCAS-dnFGFR3 resulted in the absence of, and not delayed, osteogenesis of the mandibular bones. Scale bars = 1 mm. MC, Meckel's cartilage; Cb, ceratobranchial hyoid cartilage.

Table 1. **Close correlation between the severity of mandibular outgrowth and length of Meckel's cartilage**

	Length of Meckel's cartilage (mm)
Uninjected (n = 8)	8.29 ± 0.10
RCAS (n = 8)	8.26 ± 0.11
Mild deviation (n = 3)	7.92 ± 0.10
Moderate deviation (n = 3)	6.01 ± 0.57*
Severe deviation (n = 8)	3.20 ± 0.35*

Values represent mean ± SE.
*$p < 0.01$.
The right halves of the mandibles in chick embryos between stages 17 and 20 were injected with RCAS-dnFGFR3 or control virus (RCAS). All embryos were harvested at stage 37 and processed for whole mount skeletal preparation. The length of Meckel's cartilage stained with Alcian blue was measured as described in *Materials and methods*

defects in the growth of Meckel's cartilage, the patterns of expression of *Sox9* and *Noggin* (markers of chondrogenic condensation) and *Aggrecan* (marker of overt chondrogenesis) were examined. *In situ* hybridization analysis at 4 days after injection was consistent with the formation of a truncated Meckel's cartilage on the injected side (Fig. 4A–C). While rostral expression of *Sox9*, *Aggrecan*, and *Noggin* was not present on the injected side, caudal expression was comparable with the uninjected side.

Stage-dependent effects of RCAS-dnFGFR3 on mandibular osteogenesis

Skeletal staining at stage 37 of the heads injected with RCAS-dnFGFR3 showed that all embryos also displayed

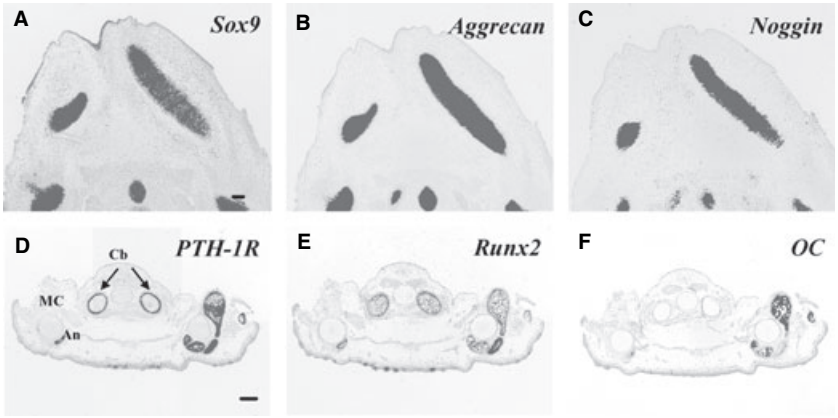


Fig. 4. Changes in the expression of markers of various stages of chondrogenesis and osteogenesis by dnFGFR3. Expression of *Sox9* (A), *Aggrecan* (B), and *Noggin* (C) in adjacent serial sagittal sections 4 days after injection of RCAS-dnFGFR3 at stage 17/18. In all pictures, the injected side of the mandible is on the left. Note the significant reductions in the domain of expression of *Sox9*, *Aggrecan* and *Noggin* on the injected sides. The magnification of all micrographs is identical. Scale bar = 100 μm Expression of *PTH-1R* (D), *Runx2* (E) and *OC* (F) in the angular bone in adjacent serial cross sections 6 days after injection of RCAS-dnFGFR3 into the right half of the mandible at stage 26. In all pictures, the injected side of the mandible is on the left. (D–F) Note the consistently smaller expression domains of *PTH-1R*, *Runx2*, and *OC* in the angular bone on the injected side adjacent to Meckel's cartilage compared to the uninjected side. No expression of *Runx2* expression was detected in the osteoblasts of the angular bone on the injected side in all sections. Note expression of *PTH-1R* and *Runx2* in the ceratobranchial cartilage undergoing endochondral ossification. The magnification of all micrographs is identical. Scale bar = 200 μm.

abnormalities (truncation and deletion/absence) in five of the mandibular bones on the injected sides (Fig. 3). The severity of abnormalities (truncation vs. deletion) in the five affected bones was stage and region dependent (Fig. 3).

Injections between stages 17 and 23 led to deletions of five bones (articular, angular, supra-angular, dentary and splenial) in a high percentage of embryos (*c.* 73–95%) (Fig. 3B, C, E–G). Abnormalities in mandibular osteogenesis were confirmed by MicroCT analysis of embryos injected with RCAS-dnFGFR3 at stage 17/18 (Fig. 3E–G). Morphometric analysis revealed 100% and 86% reductions of bone volume on the injected side compared with uninjected sides. Furthermore, absence of mandibular bones in the injected sides of embryos harvested at stage 40 (E14, 11 days after injection at stage 17/18) indicated that blocking FGFR3 signaling resulted in the absence of, and not delayed, mandibular osteogenesis (Fig. 3H).

Injection at stage 26 led to truncations of five mandibular bones on the injected sides in all embryos (Fig. 3D). This treatment led to absence of bones in a region-dependent manner in that the splenial bone displayed the highest frequency of deletion followed by deletions in the dentary and supra-angular and angular bones (Fig. 3).

To further understand the mechanism underlying the absence and truncation of mandibular bones following injection of RCAS-dnFGFR3 at stage 17/18 and 26, we analyzed the expression of early (*PTH-1R* and *Runx2*) and late (*Osteocalcin, OC*) markers of osteoblast differentiation in the angular bone as it was easily identifiable in cross sections and was consistently affected. There were decreases in the domain of expression of these markers on the injected side 6 days after injection at stage 26 (Fig. 4D–F).

Discussion

Our results show that infection of the mandibular processes with RCAS-dnFGFR3 resulted in defects in the outgrowth of Meckel's cartilage and osteogenesis of the mandibular bones in a stage- and region-dependent manner.

Injection of RCAS-dnFGFR3 into the mandibular processes between stages 17 and 20, prior to formation of the chondrogenic and osteogenic condensations, resulted in marked decreases in the outgrowth of the mandibular process, truncation of Meckel's cartilage and the absence of five out of the six mandibular bones. RCAS-dnFGFR3 did not affect the initial formation of Meckel's cartilage but led to the formation of a truncated Meckel's cartilage. These observations confirm the essential regulatory roles of Meckel's cartilage in mandibular outgrowth. Experimental studies in rodents (18, 19) showed that interference with the proper outgrowth of Meckel's cartilage in a narrow developmental

window (E14.5–E14.75) resulted in defects similar to those in Pierre-Robin sequence including a severely shortened mandible.

Our results also show that infection with RCAS-dnFGFR3 resulted in abnormalities in the developing mandibular bones in a stage- and region-dependent manner. The stage- and region-dependent effects of dnFGFR3 on mandibular osteogenesis most likely are related to infection of osteogenic cells at different stages of differentiation. Osteogenesis in the mandibular bones occurs in a caudal to rostral sequence. Ossification of bones in the caudal region (articular, supra-angular and angular) occurred at stage 31, two days prior to the ossification of bones in the more rostral region (splenial, dentary and mento-mandibular). Thus deletions of five of six bones after injection between stages 17 and 23 as well as deletion of the splenial and dentary bones after injection at stage 26 is related to the effects of dnFGFR3 on osteogenic condensation. The truncations of the caudal bones after injection after stage 26 is related to the effects of dnFGFR3 of bone forming cells at later stages of osteogenesis.

Our analysis of the angular bone showed that defects in osteogenesis were linked to changes in the levels and domains of expression of *PTH1R, OC* and *Runx2* on the injected side.

Our loss-of-function approach has provided evidence supporting the positive roles of FGFR3 during mandibular skeletogenesis. The positive roles of FGFR3 in the morphogenesis of the Meckel's cartilage is consistent with the proliferative effects of FGF signaling on proliferation of chondroblasts (20, 21). However, the most prominent abnormalities in *Fgfr3* null mutants is excessive growth of long bones through increased proliferation of the growth plate chondrocytes (22, 23). Although no bone or skull abnormalities have been described in *Fgfr3* null mutant embryos, defective bone mineralization and cortical bone osteopenia has been reported in the long bones of juvenile and young adult *Fgfr3* null mutants (24).

One possible explanation of our findings is that dnFGFR3 binds to multiple FGFRs causing a broad inhibition of FGF/FGFR signaling.

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References

1. Ornitz DM. FGF signaling in the developing endochondral skeleton. *Cytokine Growth Factor Rev* 2005;16:205–13.
2. Morriss-Kay GM, Wilkie AO. Growth of the normal skull vault and its alteration in craniosynostosis: insights from human genetics and experimental studies. *J Anat* 2005;207:637–53.
3. Itoh N, Ornitz DM. Evolution of the Fgf and Fgfr gene families. *Trends Genet* 2004;20:563–9.
4. Nie X, Luukko K, Kettunen P. FGF signalling in craniofacial development and developmental disorders. *Oral Dis* 2006;12:102–11.
5. Wilke TA, Gubbels S, Schwartz J, Richman JM. Expression of fibroblast growth factor receptors (FGFR1, FGFR2, FGFR3) in the developing head and face. *Dev Dyn* 1997;210:41–52.
6. Erceg I, Tadic T, Kronenberg MS, Marijanovic I, Lichtler AC. Dlx5 regulation of mouse osteoblast differentiation mediated by avian retrovirus vector. *Croat Med J* 2003;44:407–11.
7. Ferrari D, Harrington A, Dealy CN, Kosher RA. Dlx-5 in limb initiation in the chick embryo. *Dev Dyn* 1999;216:10–5.
8. Morgan BA, Fekete DM. Manipulating gene expression with replication-competent retroviruses. *Methods Cell Biol* 1996;51:185–218.
9. Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. 1951. *Dev Dyn* 1992;195:231–72.
10. Wang YH, Rutherford B, Upholt WB, Mina M. Effects of BMP-7 on mouse tooth mesenchyme and chick mandibular mesenchyme. *Dev Dyn* 1999;216:320–35.
11. Mina M, Wang YH, Ivanisevic AM, Upholt WB, Rodgers B. Region- and stage-specific effects of FGFs and BMPs in chick mandibular morphogenesis. *Dev Dyn* 2002;223:333–52.
12. Pizette S, Niswander L. BMPs negatively regulate structure and function of the limb apical ectodermal ridge. *Development* 1999;126:883–94.
13. Healy C, Uwanogho D, Sharpe PT. Regulation and role of Sox9 in cartilage formation. *Dev Dyn* 1999;215:69–78.
14. Sai S, Tanaka T, Kosher RA, Tanzer ML. Cloning and sequence analysis of a partial cDNA for chicken cartilage proteoglycan core protein. *Proc Natl Acad Sci U S A* 1986;83:5081–5.
15. Vortkamp A, Lee K, Lanske B, Segre GV, Kronenberg HM, Tabin CJ. Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science* 1996;273:613–22.
16. Stricker S, Fundele R, Vortkamp A, Mundlos S. Role of Runx genes in chondrocyte differentiation. *Dev Biol* 2002;245:95–108.
17. Neugebauer BM, Moore MA, Broess M, Gerstenfeld LC, Hauschka PV. Characterization of structural sequences in the chicken osteocalcin gene: expression of osteocalcin by maturing osteoblasts and by hypertrophic chondrocytes in vitro. *J Bone Miner Res* 1995;10:157–63.
18. Diewert VM. Craniofacial growth during human secondary palate formation and potential relevance of experimental cleft palate observations. *J Craniofac Genet Dev Biol Suppl* 1986;2:267–76.

19. Ricks JE, Ryder VM, Bridgewater LC, Schaalje B, Seegmiller RE. Altered mandibular development precedes the time of palate closure in mice homozygous for disproportionate micromelia: an oral clefting model supporting the Pierre-Robin sequence. *Teratology* 2002;65:116–20.
20. Iwata T, Li CL, Deng CX, Francomano CA. Highly activated Fgfr3 with the K644M mutation causes prolonged survival in severe dwarf mice. *Hum Mol Genet* 2001;10:1255–64.
21. Iwata T, Chen L, Li C, Ovchinnikov DA, Behringer RR, Francomano CA et al. A neonatal lethal mutation in FGFR3 uncouples proliferation and differentiation of growth plate chondrocytes in embryos. *Hum Mol Genet* 2000;9:1603–13.
22. Colvin JS, Bohne BA, Harding GW, McEwen DG, Ornitz DM. Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. *Nat Genet* 1996;12:390–7.
23. Deng C, Wynshaw-Boris A, Zhou F, Kuo A, Leder P. Fibroblast growth factor receptor 3 is a negative regulator of bone growth. *Cell* 1996;84:911–21.
24. Marics I, Padilla F, Guillemot JF, Scaal M, Marcelle C. FGFR4 signaling is a necessary step in limb muscle differentiation. *Development* 2002;129:4559–69.