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DEVELOPMENTAL BIOLOGY

Developmental Biology 264 (2003) 522–536

www.elsevier.com/locate/ydbio

Fgf signalling is required for formation of cartilage in the head

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Received for publication 18 December 2002, revised 12 August 2003, accepted 12 August 2003

Abstract

Characterisation of human craniofacial syndromes and studies in transgenic mice have demonstrated the requirement for Fgf signalling during morphogenesis of membrane bone of the cranium. Here, we report that Fgf activity is also required for development of the oro-pharyngeal skeleton, which develops first as cartilage with some elements subsequently becoming ossified. We show that inhibition of FGF receptor activity in the zebrafish embryo following neural crest emigration from the neural tube results in complete absence of neurocranial and pharyngeal cartilages. Moreover, this Fgf signal is required during a 6-h period soon after initiation of neural crest migration. The spatial and temporal expression of *Fgf3* and *Fgf8* in pharyngeal endoderm and ventral forebrain and its correlation with patterns of Fgf signalling activity in migrating neural crest makes them candidate regulators of cartilage development. Inhibition of Fgf3 results in the complete absence of cartilage elements that normally form in the third, fourth, fifth, and sixth pharyngeal arches, while those of the first, second, and seventh arches are largely unaffected. Inhibition of Fgf8 alone has variable, but mild, effects. However, inhibition of both Fgf3 and Fgf8 together causes a complete absence of pharyngeal cartilages and the near-complete loss of the neurocranial cartilage. These data implicate Fgf3 and Fgf8 as key regulators of cartilage formation in the vertebrate head.

Keywords: Fgf; Cartilage; Zebrafish; Embryo; Pharyngeal arch; Endoderm; Neurocranium; Neural crest

Introduction

Craniofacial syndromes account for approximately half of all human birth defects, and many of these involve abnormalities in the head skeleton (Meikle, 2002). The majority of cranial bones and all pharyngeal (viscerocranial) bones and cartilages are neural crest derivatives (Couly et al., 1993; Kontges and Lumsden, 1996; Le Douarin, 1999). By contrast, trunk skeleton is of mesodermal rather than neural crest origin (Le Douarin, 1999; Maderson, 1987).

It is now clear that abnormal signalling during development of the head skeleton underlies a growing number of human syndromes. In particular, the correct activities of fibroblast growth factor (FGF) signalling pathways are central to normal skeletal development. Mis-sense mutations in fibroblast growth factor receptors (FGFRs) underlie 11 different human skeletal dysmorphology syndromes, and several of these affect the cranial skeleton (Apert Syndrome, Beare-Stevenson Syndrome, Crouzon Syndrome, Jackson-Weiss Syndrome, and Pfeiffer Syndrome; Ornitz and Marie, 2002; Passos-Bueno et al., 1999). In addition, cultured cranial crest cells can be induced to form first cartilage and, in older cultures, to form both endochondral and membrane bone by exposure to FGF or by FGFR activation (Petiot et al., 2002; Sarkar et al., 2001).

The conserved expression in all vertebrate classes of several members of the FGF family in pharyngeal pouch endoderm and cleft ectoderm, most notably the endoderm, suggests a possible source of FGF signals that might regulate development of pharyngeal skeletal derivatives, including the jaw (Christen and Slack, 1997; Crossley and Martin, 1995a; Drucker and Goldfarb, 1993; Heikinheimo et al., 1994; Lombardo et al., 1998; MacArthur et al., 1995; Mahmood et al., 1995b, 1996; McWhirter et al., 1997; Niswander and Martin, 1992; Ohuchi et al., 1994, 2000; Shamim and Mason, 1999; Vogel et al., 1996). Moreover, Fgf-dependent Erk activity has been reported in developing pharyngeal arches (Christen and Slack, 1999). In addition, it has recently been proposed that Fgf signals emanating from the

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doi:10.1016/j.ydbio.2003.08.010

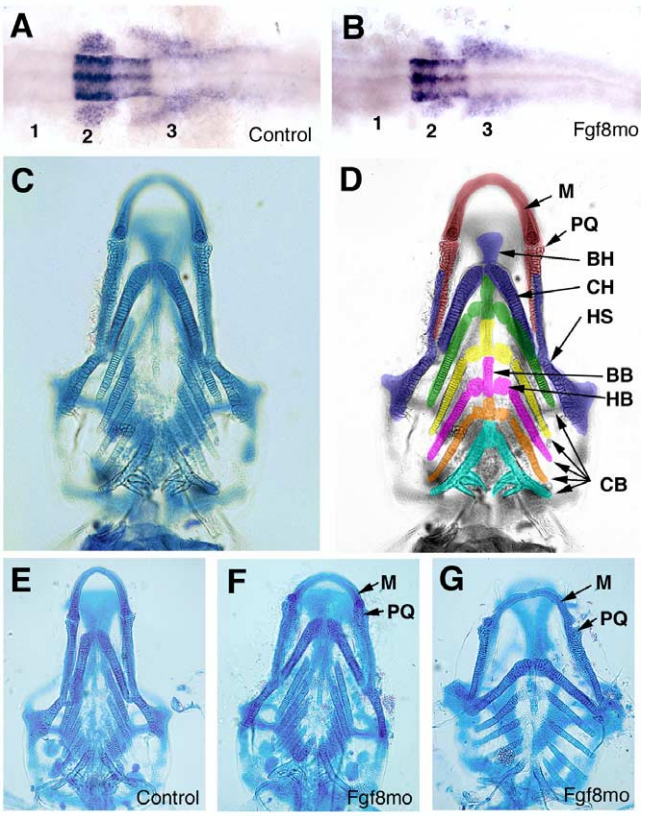

Fig. 1. Effects of inhibition of Fgf8 on *Hoxa2* expression and pharyngeal cartilage. (A, B) *Hoxa2* expression in 18-hpf embryos following injection of control morpholino (A) or Fgf8mo (B). Transcripts are detected in the hindbrain and in the second (2) and third (3) neural crest streams that will populate the second (hyoid) pharyngeal arch and posterior arches, respectively, but are not detected in cells in the position of the first (1) stream. (C) Ventral view of a wild type embryo stained for cartilage at 5 days of development. (D) The same embryo as in (C) but with the skeletal derivatives of each arch differentiated by colour and individual elements labelled: arch 1, red; arch 2, blue; arch 3, green; arch 4, yellow; arch 5, pink; arch 6, orange; and arch 7, turquoise. (E–G) Ventral views of embryos injected with the control morpholino (E) or Fgf8mo (F, G) and allowed to develop to 5 dpf, then stained for cartilage. The eyes have been removed from (C, E–F) to improve visualization of the skeleton. Abreviations: M, Meckel's cartilage: PQ, palatoquadrate; BH, basihyal; CH, ceratohyal; HS, hyosymplectic; BB, basibranchial; HB, hypobranchial; CB, ceratobranchials.

neural tube might also influence neural crest development; specifically that Fgf8 from the isthmus might repress *Hoxa2* expression in first arch crest, thereby specifying it to form first arch skeletal elements (Trainor et al., 2002).

The zebrafish offers a favourable system in which to study the regulation of head skeleton development, and several lines carrying mutations that affect pharyngeal and neurocranial cartilage formation have been identified (Kimmel et al., 1995; Neuhauss et al., 1996; Piotrowski et al., 1996; Schilling and Kimmel, 1994, 1997; Schilling et al., 1996a,b). Here, we examine the effects of inhibition of Fgf function during early zebrafish development on formation of cartilage elements. We show that inhibition of Fgf signalling results in the absence of all pharyngeal and neurocranial cartilages. Using morpholino oligonucleotides to inhibit individual members of the family, we demonstrate that Fgf3 is required for the formation of all cartilage elements derived from pharyngeal arches 3–6. More dramatically, while inhibition of Fgf8 alone had little effect on cartilage formation, inhibition of both Fgf3 and Fgf8 together resulted in near-complete absence of cranial and pharyngeal cartilages.

Materials and methods

Fish strains, microinjection of oligonucleotides, and incubation with FGFR inhibitors

Zebrafish, *Danio rerio*, of the King's wild type (kwt) strain were used throughout these studies. They were maintained at 28°C, and embryos were staged according to Kimmel et al. (1995). Morpholino oligonucleotides (GeneTools Inc.) were dissolved in distilled water at a concentration of 20 μg/ml and generally diluted to either 6 μg/ml in 120 mM KCL, 20 mM Hepes, pH 7.5, 0.25% (w/v) phenol red prior to injection. Oligonucleotides were injected in a volume of 5 nl into the yolk cell of one- to four-cell embryos just beneath the animal cell(s) as described (Holder and Xu, 1999).

Oligonucleotide sequences were as follows: Fgf3 morpholino: 5′-CATTGTGGCATGGCGGGATGTCGGC-3′; Fgf3 control morpholino: 5′-CATT**A**TG**T**CATGGCGG-GA**G**GT**G**GGC-3′; Fgf8 morpholino: 5′-GAGTCTCAT-GTTTATAGCCTCAGTA-3′; Fgf8 control morpholino: 5′-GAGT**A**TCA**G**GTTTATAG**A**CT**A**AGTA-3′.

Following injection, embryos were collected in aquarium water containing methylene blue (Westerfield, 1995) and grown at 28°C in petri dishes to the desired developmental stages.

For incubation with the FGFR inhibitor, SU5402 (Calbiochem), embryos were allowed to develop in aquarium water containing methylene blue until the 12-somite stage and were then dechorionated by incubation in 1 mg/ml pronase (Sigma) in aquarium water for 7 min. Chorions were removed by gently swirling the embryos in several changes of aquarium water, which also served to remove the pronse by serial dilution. Embryos were then incubated in either 25 or 50 μM SU5402 diluted in aquarium water from a 10 mM stock solution dissolved in DMSO for 24 h. Control embryos had an equivalent volume of DMSO added to the aquarium water. Following incubation, embryos were washed gently in several changes of aquarium water and then grown until 4 days old.

In situ hybridisation, cartilage staining, and immunohistochemistry

Embryos were dechorionated as above. In situ hybridisation using NBT/BCIP was performed as described

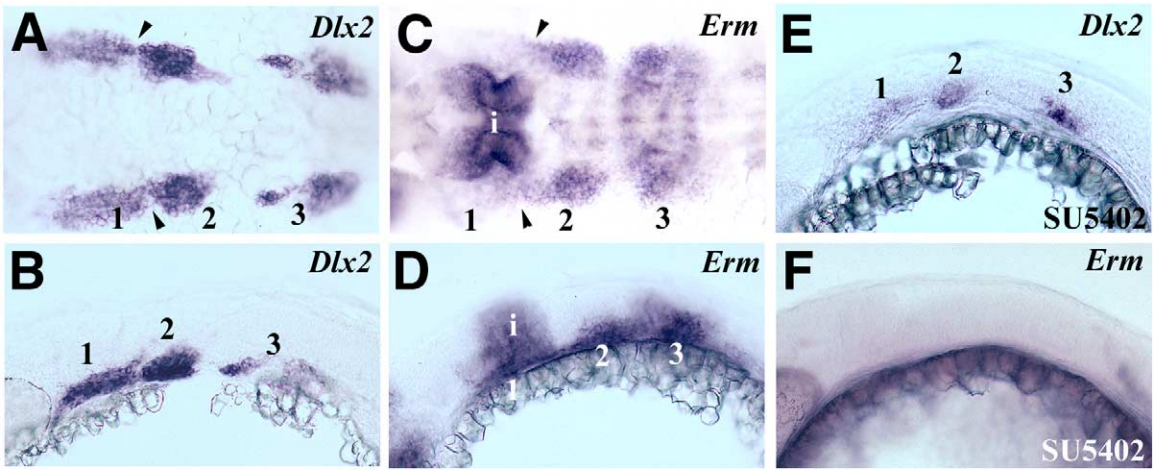

Fig. 2. Migrating neural crest cells are recipients of Fgf signals. (A, B) Dorsal (A) and lateral (B) views of 24-hpf embryos showing expression of *Dlx2* to show the locations of the three pharyngeal neural crest streams (numbered). (C, D) Dorsal (C) and lateral (D) views of 24-hpf embryos showing expression of *Erm* in the second and third neural crest streams but little expression in the first stream that migrates adjacent to the isthmus. Transcripts are also detected in the neural tube associated with the isthmus (i) and are detected more weakly in rhombomere boundaries in (C). (E, F) Treatment of embryos with 100 μM SU5402 between 18 and 24 hpf: *Dlx2* expression shows that all three neural crest streams are still present (E) but *Erm* is undetectable (F). Arrowheads in (A) and (C) indicate the position of the first pharyngeal pouch.

(Shamim et al., 1999; Irving and Mason 2001). For colour reactions using p-iodonitrotetrazolium violet (Sigma) as the second substrate, embryos were incubated in 0.188 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate and 0.2 mg/ml p-iodonitrotetrazolium violet (Liang et al., 2000). Alcian blue staining for cartilage in whole embryos was performed as described (Schilling et al., 1996b). For immunocytochemistry, embryos were fixed for 3 h in 2% trichloroacetic acid in H_2O, washed in H_2O, then incubated in acetone for 20 min at −20°C. After washes in H_2O and PBS, embryos were blocked in 10% DMSO/1% BSA/2% goat serum/0.1% Triton X-100/PBS for several hours before incubation with mouse anti-neurofilament-160kD (Zymed, 13-0700) at 1:10,000 dilution in the same mixture for 48 h. Embryos were then washed for several hours in several changes of PBST (0.1% Triton X-100/PBS) and then incubated overnight with goat anti-mouse HRP (Sigma, A8924) in PBST. After several washes in PBST, the colour reaction was performed by using reagents provided in the DAB Liquid Substrate System kit (Sigma, D-7304).

Detection of cell death

Apoptotic cells were detected by using the DeadEnd colourimetric detection kit (Promega). Embryos were dechorionated and fixed overnight in 4% w/v paraformaldehyde in PBS. They were then incubated in methanol for 30 min and rehydrated through a graded series of methanol (75%, 50%, 25%) in PBST (PBS, 0.1% Tween 20), washed twice in PBST, and twice in PBS. They were incubated in proteinase K (5 μg/ml in PBS) for exactly 5 min, washed in PBS, and postfixed in 4% w/v paraformaldhyde in PBS for 20 min. The embryos were then washed three times in PBS and three times in acetone, being stored at −20°C for 10

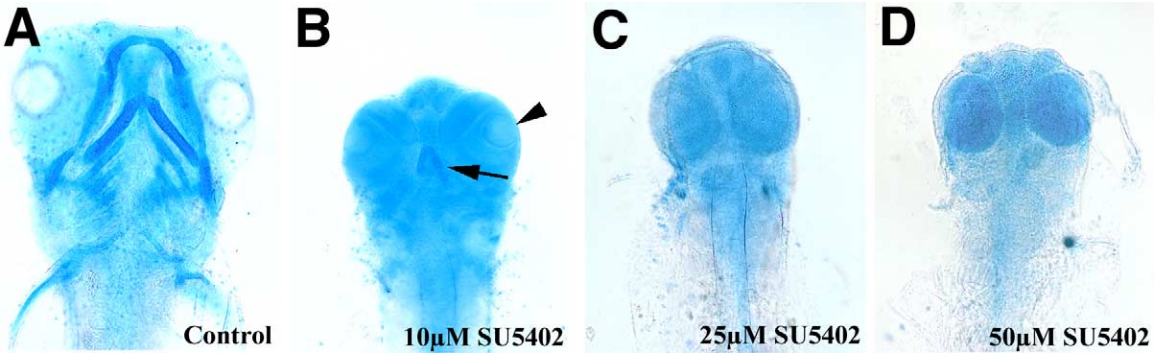

Fig. 3. Fgf signalling is required for formation of pharyngeal and viscerocranial cartilages. (A–D) Ventral view of 4-dpf embryos stained to visualise cartilage following exposure to SU5402 for 24 h from the 12-somite stage (15 hpf). (A) Control embryo. (B–D) Embryos exposed to 10, 25, and 50 μM SU5402, respectively. A single, small cartilage element (arrow) is present in (B), and lenses are also present in that specimen (arrowhead) but these are absent from (C) and (D).

min in the final acetone wash. They were rinsed three times in PBS before being incubated for 10 min with equilibration buffer (DeadEnd kit). The equilibration buffer was removed and replaced with the terminal transferase reaction mix containing biotinylated UTP (DeadEnd kit), and the reaction was incubated at 37°C for 3 h. Embryos were washed in 2× SSC for 30 min, three times in PBST, and three times in PBS before being incubated in streptavidin–HRP 1/500 in PBS for 1 h. After three washes in PBS, the colour reaction was performed by using the DAB substrate and reaction mix provided with the kit. The reaction was stopped with several washes in distilled water, then in PBS, and embryos were cleared in 90% glycerol, PBS.

Sectioning

Following in situ hybridisation, embryos were embedded in Technovit 8100 resin according to the manufacturer's instructions (Heraeus, Technovit 8100). Sections of 8.5-μm thickness were cut by using a Reichert-Jung manual microtome (1140/Autocut) and mounted on slides. Sections were counterstained with nuclear fast red then permanently mounted under a coverslip using DPX mountant (BDH, 360292F).

Results

Evidence from ectopic expression and loss-of-function studies in all vertebrate classes indicates that Fgf8 from the isthmus (midbrain–hindbrain boundary) provides a patterning signal for adjacent midbrain and anterior hindbrain (Crossley et al., 1996; Hunter et al., 2001; Irving and Mason, 1999, 2000; Lee et al., 1997; Liu et al., 1999; Martinez et al., 1999; Meyers et al., 1998; Picker et al., 1999; Reifers et al., 1998; Shamim et al., 1999). Moreover, ectopic expression and complimentary inhibition experiments in chick embryos show that Fgf8 establishes the anterior limit of *Hox* transcripts within the hindbrain by repression of *Hoxa2* to define the boundary between rhombomeres 1 and 2 (Irving and Mason, 2000). However, much less is known about isthmic Fgf8 effects on tissues adjacent to that region of the brain. Neural crest cells that populate the first pharyngeal arch and migrate close to the isthmus do not express *Hoxa2* in any vertebrate, whereas those populating all other arches do (Hunter and Prince, 2002; Prince and Lumsden, 1994; Prince et al., 1998; Schilling et al., 2001; Trainor et al., 2002; Trainor and Krumlauf, 2001, and references therein). It was recently shown that ectopic Fgf8 could transiently repress *Hoxa2* expression in neural crest migrating from the hindbrain, prompting the authors to suggest a role for isthmic Fgf8 in specification of first arch neural crest (Trainor et al., 2002). A prediction of that study is that, in the absence of isthmic Fgf signals, first arch neural crest would express *Hoxa2* and form cartilage elements characteristic of posterior arches.

To further investigate the possible role of Fgf8 from the isthmus on specification and development of first arch neural crest, we examined the effect of loss of Fgf8 function in zebrafish. We and others have previously shown that the *acerebellar (ace)* zebrafish mutant, which lacks Fgf8 function (Brand et al., 1996; Picker et al., 1999; Reifers et al., 1998; Shanmugalingam et al., 2000), can be exactly phenocopied following injection of antisense oligonucleotides (morpholinos or morphants) into one- to four-cell-stage embryos (Araki and Brand, 2001; Draper et al., 2001; Maroon et al., 2002; Walshe et al., 2002; Walshe and Mason, 2003).

The effects of loss of Fgf8 function on *Hoxa2* expression in neural crest were examined in zebrafish embryos injected with either Fgf8mo or a control morpholino (the Fgf8mo sequence but with 4 single, nonclustered nucleotide changes; Maroon et al., 2002). Embryos were allowed to develop until 18 h postfertilization (hpf) when the emergent crest streams are migrating adjacent to the hindbrain. Embryos injected with Fgf8mo showed no change in expression of *Hoxa2* (n = 20/20, two experiments; Fig. 1B) when compared with those injected with the control morpholino (n = 22/22, two experiments; Fig. 1A) or uninjected embryos (data not shown; Hunter and Prince, 2002; Prince et al., 1998; Schilling et al., 2001). In particular, no ectopic transcripts were detected in the first arch crest stream, the position of which can be determined by hybridisation for *Dlx2* (see Figs. 2A and B, and 4A–H).

It remained possible, however, that low levels of *Hoxa2* were induced in the first arch crest and/or that it would be incorrectly specified for other reasons in embryos injected with Fgf8mo. In either case, it would be expected that skeletal development would be severely affected. Loss of isthmic Fgf8 function in both *ace* fish or following injection of Fgf8 morpholinos (Fgf8mo) is readily detected at 30 hpf by the complete absence of the cerebellum, which forms posterior and adjacent to the isthmus (Brand et al., 1996; Maroon et al., 2002; Reifers et al., 1998). Zebrafish embryos, injected with Fgf8mo, were examined at that developmental stage. Those lacking all cerebellar structures were selected and were allowed to develop further until 5 days postfertilization (dpf) when cartilage was visualised in the whole embryos by Alcian blue staining of proteoglycans. The cartilage derivatives of each of the seven pharyngeal arches are readily identifiable at 5 dpf (Schilling et al., 1996a,b) and those of arches 1, 2, and 7 are individually distinct, the latter bearing pharyngeal teeth (Fig. 1C and D). Zebrafish injected with Fgf8mo (n = 34/34) showed no evidence of respecification to more posterior arch structures (compare Fig. 1F and G with Fig. 1C and E). In particular, both of the distinctive first arch elements, Meckel's cartilage and the palatoquadrate, were clearly present (Fig. 1F and G). However, in a subset of embryos (n = 6/34), the first arch skeletal elements were somewhat dysmorphic (Fig. 1G) when compared with uninjected embryos or to those

injected with control morpholino, and in most embryos the jaw was slightly shorter and wider.

Since the studies of Fgf8 found little evidence for a function for that protein in pharyngeal crest skeletogenic development, we sought to determine whether or not these neural crest cells were normally subject to Fgf signals. Expression of the Erk-inducible transcription factors, Erm and Pea3, were recently shown to be completely Fgf-dependent during early stages of zebrafish development (Maroon et al., 2002; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001; Walshe et al., 2002). During crest migration, *Erm* transcripts were strongly detected in streams migrating from the hindbrain to populate the second and third pharyngeal arches (compare Fig. 2A and B with C and D). By contrast, very little *Erm* expression was detectable in the first arch stream adjacent to the isthmus, although strong expression was detected in the mid–hindbrain region of the neural tube itself (Fig. 2C and D), and transcripts were detected in first arch mesenchyme only at later stages (30 hpf; data not shown). The latter observation provides further evidence that isthmic Fgf signalling does not greatly influence first arch neural crest development. Indeed, those *Erm* transcripts detected in the first arch crest were in cells adjacent to the first pharyngeal pouch rather than the isthmus (Fig. 2C).

To confirm that the *Erm* expression detected in posterior cranial neural crest streams was dependent on Fgf signalling, embryos were exposed to SU5402, an inhibitor of FGF receptor activation, between 18 and 24 hpf. This resulted in the complete loss of *Erm* expression in both neural crest and the neuroepithelium (Fig. 2F), while expression of the neural crest marker, *Dlx2*, although reduced, showed that all three crest streams were still present (Fig. 2E). These data indicated that the second and third pharyngeal crest streams were normally recipients of Fgf signals soon after emigration from the hindbrain.

To determine whether or not this Fgf signalling was required for pharyngeal skeletal development, embryos were incubated in SU5402 at a range of concentrations for 24 h following the onset of neural crest migration at the 12-somite stage (about 15 hpf). Embryos were washed extensively to remove residual inhibitor and then allowed to develop until 4 dpf, when they were stained for cartilage formation. Even at the lowest concentrations of SU5402 used in this study, all cartilage elements of the pharyngeal skeleton and neurocranium were missing with the exception of a small element, possibly rudimentary trabeculae cranii of the neurocranium, just anterior to the notochord (n = 11/11; Fig. 3A and B). The presence of lenses in the eyes of these embryos suggested that Fgf activity had not been abolished completely as lens induction is Fgf-dependent. Embryos exposed to higher concentrations of SU5402 lacked all cartilage elements and lenses were also absent (n = 8/8 at 25 μM and 5/5 at 50 μM; Fig. 3C and D). These results indicated that Fgf signalling was required for the development of both neurocranial and pharyngeal arch cartilage.

A candidate for that signal was Fgf3, expressed by pharyngeal ectoderm and endoderm, most notably in the endodermal pouches, in other vertebrates (Lombardo et al., 1998; Mahmood et al., 1995b, 1996). At the onset of neural crest migration (12 somites; 15 hpf), *Fgf3* was detected in hindbrain rhombomere 4 and at the isthmus, although transcripts were lost from rhombomere 4 after 20 hpf (Fig. 4A–H; Maroon et al., 2002; Walshe et al., 2002; and data not shown;). At the 14-somite stage (16 hpf), transcripts were also in the endoderm of the first pharyngeal pouch located between arches 1 and 2 (data not shown). Shortly thereafter, *Fgf3* mRNA was also present in the second pouch located between arches 2 and 3 (Fig. 4A–G). Subsequently, transcripts became reduced in the first pouch (Fig. 4D and H), but remained in the second pouch and appeared in the third pouch (Fig. 4C, D, G, and H). Sectioning of specimens showed that *Fgf3* expression was confined to the pouch endoderm (see Fig. 6D), and cohybridisation for *Dlx2* showed that this endoderm was closely associated with all three neural crest streams (Fig. 4A–H).

We have previously shown that antisense morpholino oligonucleotides (Fgf3mo) can inhibit translation of Fgf3, revealing roles for that protein in otic, hindbrain, and forebrain development (Maroon et al., 2002; Walshe et al., 2002; Walshe and Mason, 2003). We used the same approach to examine Fgf3 function in pharyngeal neural crest and cartilage development. *Dlx2* expression showed that all three neural crest streams were still present in embryos injected with Fgf3mo at 21 hpf and that neural crest had populated the arches at 30 hpf. However, expression was sometimes reduced at 21 hpf (n = 6/17 normal expression; n = 11/17 reduced expression in all three streams) when compared with embryos injected with a control morpholino (Fgf3mo with 4 nonclustered single nucleotide substitutions, see Maroon et al., 2002; Fig. 4I–L). Following inhibition of Fgf3, *Erm* was still expressed by mesenchymal cells adjacent to the hindbrain, including those in the positions of the *Dlx2*-positive neural crest population (n = 17/17; Fig. 4M–P), but transcripts were not detected in the posterior part of the third stream, which populates the third and posterior arches (compare Fig. 4N with P). In addition, the second and third neural crest streams were sometimes less clearly defined following Fgf3mo injection, possibly due to abnormal development of the otic vesicle, which normally serves to separate migrating crest streams (Maroon et al., 2002; Nissen et al., 2003). We found no evidence for increased cell death in migrating neural crest in embryos injected with Fgf3mo; however, there was evidence of increased apoptosis in the dorsal neural tube, particularly at spinal cord levels (Fig. 4Q–T) and also in the tailbud (data not shown; Walshe et al., 2002; Walshe and Mason, 2003).

The effect of inhibition of Fgf3 on cartilage development was examined in embryos allowed to develop to 5 dpf. At

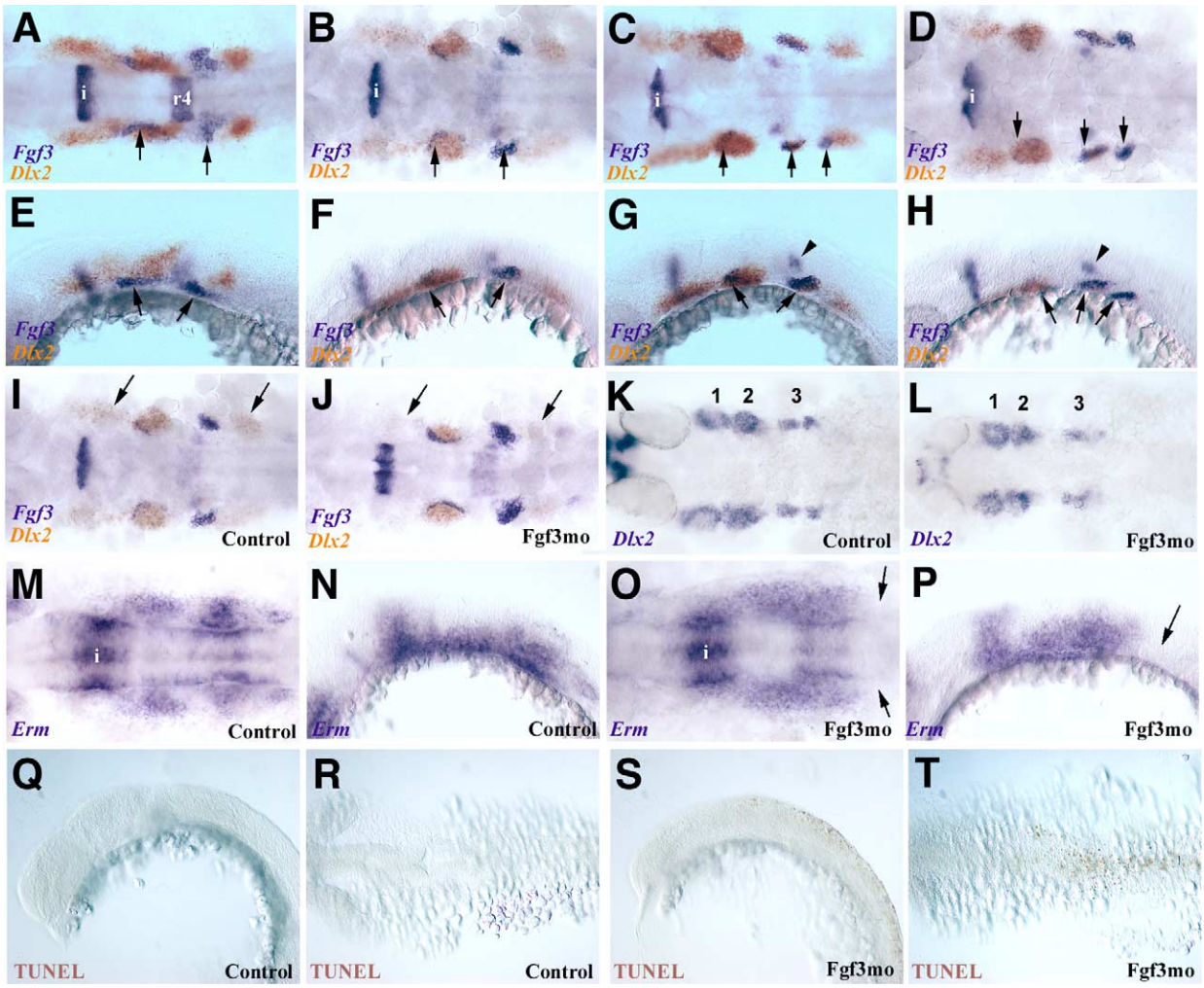

Fig. 4. *Fgf3* expression by pharyngeal pouch endoderm closely associated with neural crest streams. (A–H) Close relationship of endodermal *Fgf3* expression to neural crest streams shown by hybridisation for *Fgf3* (blue) and *Dlx2* (orange) transcripts. Dorsal (A–D) and lateral (E–H) views of 18 (A, E), 21 (B, F), 24 (C, G), and 28 hpf (D, H) embryos. *Fgf3* is expressed in endoderm of the first, second, and third pharyngeal pouches (arrows), also the isthmus (i) and rhombomere 4 (r4) in younger embryos (A, B, E, F) and in the anterior of the otic vesicle in older embryos (B–D, F–H; arrowhead in G, H). (I–L) *Dlx2*-positive neural crest cells are still present in embryos at 21 and 30 hpf following inhibition of Fgf3 translation with morpholinos. In situ hybridisation for *Dlx2* (orange) and *Fgf3* (blue) transcripts in embryos at 21 hpf injected with a control morpholino (I; dorsal view) or Fgf3mo (J; dorsal view). Arrows indicate first and third neural crest streams. Dlx2 expression at 30 hpf in embryos injected with a control morpholino (K; dorsal view) or Fgf3mo (L; dorsal view). (M–P) Posterior but not anterior expression of *Erm* by neural crest cells in 18-hpf embryos is reduced following inhibition of Fgf3. Dorsal (M, O) and lateral (N, P) views of embryos injected with control morpholino (M, N) or Fgf3mo (O, P). *Erm* transcripts are not detected in the most posterior of the neural crest streams (arrows). Isthmic *Erm* expression (i) is indicated in (M) and (O). (Q–T) Cell death is not detected in migrating crest streams at 20 hpf following inhibition of Fgf3. Dorsal (Q, S) and lateral (R, T) views of embryos injected with control (Q, R) or Fgf3mo oligonucleotides and analysed by TUNEL staining. No apoptotic cells are apparent in control embryos (Q, R), while death is restricted to dorsal neural tube at hindbrain and cord levels following inhibition of Fgf3 (S, T).

lower concentrations of Fgf3mo (5 μg/μl), all cartilage derivatives of the first and second arches were present and identifiable but somewhat dysmorphic. Most notably, the ceratohyal elements projected posteriorly rather than anteriorly; however, this did not represent a reversal in second arch polarity as the midline basihyal elements still projected anteriorly (Fig. 5C and D). The seventh ceratobranchial cartilage, readily identifiable by the pharyngeal teeth that it bears, was present and apparently normal. However, the ceratobranchial elements derived from arches 3–6 were greatly reduced in size when compared with embryos injected with the control morpholino (n = 14/16; Fig. 5A and B).

At higher concentrations of Fgf3mo (6 μg/μl) ceratobranchial, basibranchial, and hypobranchial elements derived from arches 3–6 were completely absent with just a small, midline cartilage nodule remaining in some specimens. In addition, first and second arch elements were all present but were more severely deformed than with the lower concentration of Fgf3mo, but the seventh arch ceratobranchial element remained unaffected as did neurocranial cartilage (n = 22/25; Fig. 5C and D).

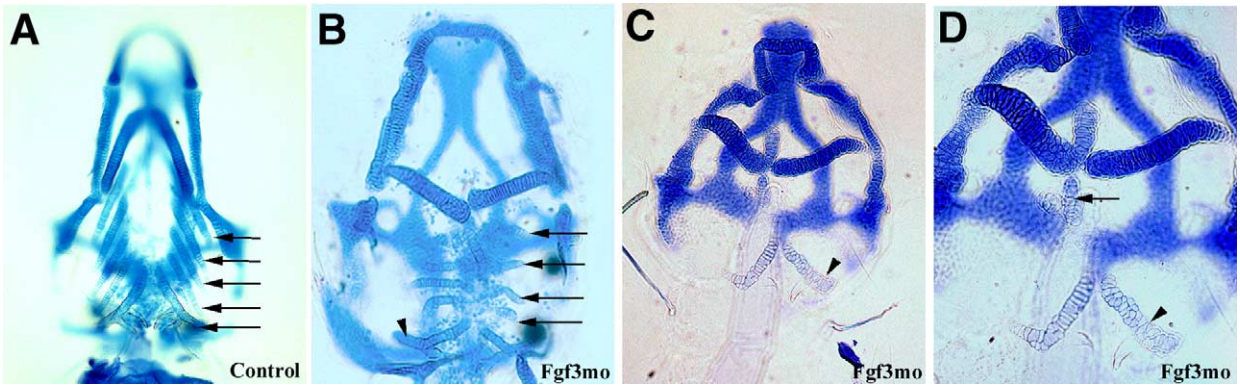

Fig. 5. Fgf3 is required for formation of ceratobranchial cartilages. (A–D) Ventral views showing loss of cartilage derivatives of pharyngeal arches 3-6 in 5-dpf embryos following inhibition of Fgf3. (A) Embryo injected with control morpholino. (B) Embryo injected with low concentration of Fgf3mo shows reduction in ceratobranchial cartilages derived from arches 3-6 (arrows). (C, D) Low and high magnification images of an embryo injected with a higher concentration of Fgf3mo. There is a complete absence of all ceratobranchial cartilage derivatives of arches 3–6. Hypobranchial and basibranchial elements are also missing, although a small midline cartilage nodule is present (arrow in T). However, the tooth-bearing ceratobranchial element derived from arch 7 is unaffected (arrowheads in B–D).

These data indicate that Fgf3 is absolutely required for the development of all cartilage derivatives of the third, fourth, fifth, and sixth pharyngeal arches. However, the effects of Fgf3 inhibition only partially recapitulated the complete loss of cartilage observed after treatment with SU5402. This suggested that other Fgfs might act together with Fgf3 in regulating cartilage development in the first, second, and seventh arches. We and others have shown that Fgf8 acts in combination with Fgf3 to direct otic, hindbrain, and forebrain development (Leger and Brand, 2002; Maroon et al., 2002; Maves et al., 2002; Phillips et al., 2001; Walshe et al., 2002; Walshe and Mason, 2003). We therefore examined *Fgf8* expression during the period of neural crest migration and pharyngeal arch formation. At early stages of neural crest migration, *Fgf8* was detected in endoderm adjacent to the hindbrain (Fig. 6A–C) and, at later stages exclusively in endoderm immediately anterior to the first arch (Fig. 6E and F), where it was coexpressed with *Fgf3* (Fig. 6G and H).

Fgf3 and Fgf8 were inhibited together in embryos injected with both Fgf3mo (5 μg/μl) and Fgf8mo (6 μg/μl). In such embryos, three *Dlx2*-positive neural crest streams were detected at 21 hpf; however, as seen with Fgf3mo alone, the extent of *Dlx2* expression was sometimes reduced (n = 4/11) when compared with embryos injected with both control morpholinos (Fig. 6I–K). *Dlx2*-positive neural crest was also detected in the arches at 30 hpf following injection with Fgf3mo and Fgf8mo; however, expression in the posterior arches was reduced (15/19; Fig. 6L). TUNEL staining indicated that this reduction in expression was unlikely to be due to cell death in the migrating neural crest streams, although as with inhibition of Fgf3 alone, there was increased cell death in the alar spinal cord (compare Fig. 6M–P and Fig. 4Q–T; Maroon et al., 2002; Walshe et al., 2002; Walshe and Mason, 2003 and data not shown). Cell division was likewise unaffected in embryos injected with Fgf3mo, Fgf8mo, or both Fgf3mo and Fgf8mo (data not shown). It would therefore seem that the reduction in *Dlx2* expression seen when either both Fgf3 and Fgf8 are inhibited or following SU5402 treatment is unlikely to be due to loss of neural crest cells. *Dlx2* induction and/or maintenance would seem to be partly dependent on Fgf signalling; however, it has not been demonstrated that all pharyngeal neural crest cells express *Dlx2* or whether this is a property of a specific subpopulation.

Embryos allowed to develop until 5 dpf following injection with both Fgf3mo and Fgf8mo showed a dramatic loss of cartilage from the head (n = 13/15; Fig. 7). There was a complete absence of jaw elements, all derivatives of arches 3–7, and most of the neurocranial cartilage. All embryos had a small residual midline element located just anterior to the notochord, which was sometimes flanked by more lateral elements having superficial resemblance to hyosymplectic or possibly to parachordal cartilages. However, it was impossible to identify these residual elements as being either neurocranial or viscerocranial, and their morphology varied from individual to individual. These data indicate that Fgf3 acts in combination with Fgf8 to regulate cartilage development in both the pharynx and neurocranium and that their combined inhibition largely reproduces the effects of loss of all FGFR activity.

Embryos injected with Fgf morpholinos were examined by TUNEL analysis at various stages subsequent to neural crest migration to determine whether or not cell death within the arches could account for the dramatic lack of cartilage observed. Very few apoptotic cells were detected in the arches of embryos injected with control morpholinos, Fgf3mo, or both Fgf3mo and Fgf8mo at 25 hpf (Fig. 8A–C), and while more dead cells were detected in Fgf morpholino-injected embryos than in control morpholino-injected embryos at 30 hpf (Fig. 8D–F) and at 48 hpf (Fig. 8G–I), these were not specifically located to the arches.

We additionally investigated the requirement for Fgf3 and Fgf8 in the development of nonectomesenchymal (non-

cartilage-forming) neural crest populations. *Sox10* is expressed by neural crest that differentiates into neuronal, glial, and pigment cells (Dutton et al., 2001). Streams of *Sox10*-expressing cells were detected in 24-hpf embryos injected with control morpholinos, Fgf3mo (7/7), and both Fgf3mo and Fgf8mo (11/11; Fig. 8J–L). A subset of non-ectomesenchymal neural crest cells differentiate into neuronal cells of the trigeminal ganglion in chick and mouse (D'Amico-Martel and Noden, 1983; Stainier and Gilbert, 1991), and there is also strong evidence for this in zebrafish (Norton et al., 2000). We therefore examined the trigeminal ganglia in embryos at 48 hpf but could detect no defects following injection with either control moprholinos, Fgf3mo (24/24), or Fgf3mo and Fgf8mo (22/22; Fig. 8M–O). Although limited in extent, these data suggest that only the cartilage-forming subset of the total neural crest population is dependent on Fgf3 and Fgf8 for marker gene expression and differentiation.

Taken together, our data indicate that development of oro-pharyngeal cartilage elements in the zebrafish is dependent on Fgf signalling and that Fgf3 and Fgf8 are the major ligands involved in that process.

Discussion

In this study, we explored the requirement for Fgf signalling in the development of cranial neural crest and, in particular, its ability to form cartilage elements in the head. Inhibition of FGF receptor activation with the pharmacological agent, SU5402, resulted in a complete loss of pharyngeal and neurocranial cartilages. At lower concentrations of inhibitor, head cartilage was reduced to a midline element located anterior to the notochord with superficial resemblance to the neurocranial trabeculae cranii. However, the presence of lenses in these embryos suggested that not all Fgf signalling had been inhibited in these embryos; lens induction and differentiation being Fgf-dependent processes (Faber et al., 2001; Govindarajan and Overbeek, 2001, and references therein). By contrast, no lenses developed in embryos treated with higher concentrations of SU5402, and such embryos completely lacked neurocranial and pharyngeal cartilages, including jaw structures. Thus, FGF signalling is a required component in the process leading to chondrogenic differentiation by both cranial and pharyngeal neural crest populations.

We therefore investigated the roles of members of the Fgf family in regulating cartilage formation. Their expression in tissues associated with neural crest at either early (hindbrain, isthmus) or later (pharyngeal endoderm, ventral forebrain) stages of its development in zebrafish embryos made Fgf3 and Fgf8 attractive candidates for providing the signalling cue (this study; David et al., 2002; Furthauer et al., 2001; Maroon et al., 2002; Phillips et al., 2001; Raible and Brand, 2001; Reifers et al., 1998; Shanmugalingam et al., 2000; Walshe et al., 2002; Walshe and Mason, 2003). Inhibition of Fgf8 alone with morpholino oligonucleotides at a range of concentrations resulted only in no defects or occasional relatively mild and variable dysmorphology of pharyngeal cartilages. Furthermore, in such embryos, all neurocranial and pharyngeal elements were readily identifiable and thus had been correctly specified. These data are consistent with preliminary studies of the *acerebellar (ace)* zebrafish mutant (David et al., 2002; Roehl and Nusslein-Volhard, 2001), although it remains unclear whether or not *ace* is a hypomorphic or null *Fgf8* allele (see Reifers et al., 1998; Araki and Brand, 2001; Draper et al., 2001 for discussion).

By contrast, loss of Fgf3 resulted in complete absence of cartilage derivatives (ceratobranchials, basibranchials, and hypobranchials) of pharyngeal arches 3–6 and dysmorphology in derivatives of the maxillomandibular (arch 1) and hyoid (arch 2) arches. However, the fifth ceratobranchial element, derived from arch 7, was unaffected by inhibition of Fgf3. It is also noteworthy that the ceratohyoid cartilages projected posteriorly instead of anteriorly, but the basihyal elements still projected anteriorly, showing that this did not represent a reversal of second arch polarity.

Most significantly, inhibition of both Fgf3 and Fgf8 together resulted in the near-complete absence of cranial cartilage elements. Pharyngeal cartilages were completely absent, and the neurocranium was reduced to a midline cartilage located anterior to the notochord. Thus, while Fgf3 alone is sufficient to promote cartilage development in pharyngeal arches 3–6, it functions together with Fgf8 to promote chondrogenesis within the remaining three arches (1, 2, and 7) and neurocranium.

It is noteworthy that the small, residual cartilage elements present following inhibition of both Fgf3 and Fgf8 had similar position, just anterior to the notochord, and often had similar morphology to those produced when embryos were treated with the lower concentrations of SU5402 (compare Figs. 3B and 7D). Based on their position and morphology we speculate that these might be vestigial trabeculae cranii cartilages. The presence of small lenses in the eyes of embryos treated with that dose of SU5402 suggested that Fgf signalling had not been completely inhibited. Thus, the similarity between the effects of low concentrations of SU5402 and inhibition of both Fgf3 and Fgf8 may indicate that another Fgf also signals to a subpopulation of cranial crest. It is also possible that Fgf3 and/or Fgf8 had not been completely inhibited, although this seems unlikely as increasing the concentration of morpholinos injected did not result in further loss or reduction in size of the residual cartilage elements (data not shown). The location of the remaining cartilage, just anterior to the notochord, suggests that the latter might also be the source of an Fgf signal. Indeed, *Fgf4* is expressed by notochord in avian embryos (Shamim et al., 1999; Shamim and Mason, 1999), although inhibition of Fgf4 alone has no effect on cranial cartilages in zebrafish (David et al., 2002).

The complex and dynamic expression of *Fgf3* and *Fgf8*

during head development in zebrafish (this study and references above), which is largely conserved among other vertebrates (Christen and Slack, 1997; Crossley and Martin, 1995b; Mahmood et al., 1995a, b, 1996; Shamim et al., 1999; Tannahill et al., 1992; Wilkinson et al., 1988, 1989), begs the question: which are the tissue sources of Fgf3 and Fgf8 that regulate cartilage formation? In this regard, it is particularly important to note that SU5402-mediated inhibition of Fgf signalling after 15 h of development resulted in complete absence of cranial cartilage. As discussed below, this would suggest that the observed defects were unlikely to be an indirect consequence of earlier Fgf3 and Fgf8 functions in either isthmus or hindbrain.

Fgf3 and Fgf8 are transiently expressed in the presumptive rhombomere 4 territory of the developing hindbrain and are required together for both establishment of hindbrain segmental identity and concommitant induction of the otic placode (Leger and Brand, 2002; Maroon et al., 2002; Maves et al., 2002; Phillips et al., 2001; Walshe et al., 2002). The failure to establish the molecular identity of individual rhombomeres would potentially affect any specification of neural crest precursors. However, signalling is only required during a brief window of development between 80% epiboly and the 2- to 3-somite stages (11 hpf) for hindbrain patterning and otic induction; thereafter inhibition of FGFR activity with SU5402 has no effect on hindbrain development (Maroon et al., 2002; Walshe et al., 2002). By contrast, complete loss of head cartilage occurs when embryos are exposed to inhibitor from the 12-somite stage onwards (15 hpf). Thus, it seems unlikely that the effects of Fgf3 and Fgf8 on cartilage development are due to failure to correctly specify neural crest precursors prior to their migration from the hindbrain. Moreover, although Fgf3, but not Fgf8, is expressed in rhombomere 4 at the onset of crest migration (Fig. 4), there is good evidence that pharyngeal endoderm is the source of that factor which regulates cartilage development (see below).

The isthmus is a second neuroepithelial source of both Fgf3 and Fgf8 that potentially regulates neural crest development. Fgf8 signals both anteriorly and posteriorly from the isthmus to pattern midbrain and anterior hindbrain, respectively. In its absence, most of the midbrain and all derivatives of hindbrain rhombomere 1, including the cerebellum, are lost. Since Fgf8 is required to establish and/or maintain positional identity within the mid–hindbrain territory, it might also establish a prepattern for the midbrain/anterior hindbrain neural crest that migrates into the first pharyngeal arch, the facial and cranial regions. In particular, complimentary inhibition and ectopic expression studies in avian embryos have shown that Fgf8 prevents expression of *Hoxa2* in the most anterior hindbrain segment (Irving and Mason, 2000). Recent studies in the chick have shown that ectopic Fgf8 transiently inhibits *Hoxa2* expression in the neural crest stream that populates the second pharyngeal arch in the chick. Furthermore, grafts of mid–hindbrain tissue, including the isthmus to the position of rhombomere 4, resulted in the subsequent development of skeletal elements resembling first arch derivatives (Meckel's cartilage and quadrate) within the second arch. One interpretation of these data is that isthmic Fgf8 normally inhibits *Hoxa2* expression in first arch crest migrating from midbrain and anterior hindbrain, including rhombomere 2, which itself expresses *Hoxa2* (Trainor et al., 2002). This would therefore be a modification of a *Hox* code prepattern model in which *Hoxa2* expression would be repressed in neural crest by isthmic Fgf8 both prior to (midbrain and rhombomere 1) and during (rhombomere 2 crest) its early migration.

Inhibition or loss of Fgf8 function in zebrafish results in an absence of isthmic tissue as demonstrated by the loss of all mid-hindbrain and isthmic markers (Reifers et al., 1998). Notably, *Fgf3* and *Fgf17*, which are expressed in the isthmus at later stages of development and thus might compensate for loss of Fgf8 function, are not expressed in that tissue when the latter is inhibited (Reifers et al., 2000; unpublished observations). Such embryos, in which isthmic Fgf8 function had been compromised, as demonstrated by complete absence of a cerebellum at 24 hpf, were allowed to develop further and stained for cartilage formation. In these embryos, the first arch skeletal elements (Meckel's cartilage and the palatoquadrate) were always present, although variably showed some dysmorphology. In addition, *Hoxa2* transcripts were never detected in the first arch neural crest; such ectopic expression would have been expected to lead to replacement of first arch elements by second arch ones (Grammatopoulos et al., 2000; Pasqualetti et al., 2000). Thus, our studies in the zebrafish find no requirement for isthmic Fgf signalling in neural crest patterning. These data are compatible with the results of pharmacological inhibition, which show a requirement for Fgf signalling in isthmic patterning only between late epiboly and early somite stages (Maroon et al., 2002; Walshe et al., 2002; and J.W., H. Maroon and I.M., unpublished data) but a later requirement for cartilage formation. Taken together, these data indicate that failure of cranial cartilage formation following inhibition of Fgf3 alone or Fgf3 and Fgf8 together is not a consequence of signalling from sources within the neuroepithelium. This is of particular importance as it would also seem to rule out a role for neural Fgf3 and Fgf8 in establishing any prepattern within neural crest progenitors.

The most likely source of Fgfs that direct chondrogenic development in pharyngeal neural crest is pharyngeal endoderm and possibly also pharyngeal ectoderm. At or soon after the onset of neural crest migration, *Fgf8* is transiently expressed in endoderm associated with the arches, and *Fgf3* is expressed sequentially in the endodermal pouches separating the first four pharyngeal arches. At later developmental stages, both are expressed in endoderm associated with the first arch (this study; David et al., 2002). Neural crest migrating close to these tissues express the Fgf-dependent transcription factors, *Erm* and *Pea3*. It is noteworthy that only the first arch neural crest cells closest to the first pouch endoderm express *Erm*, while those cells

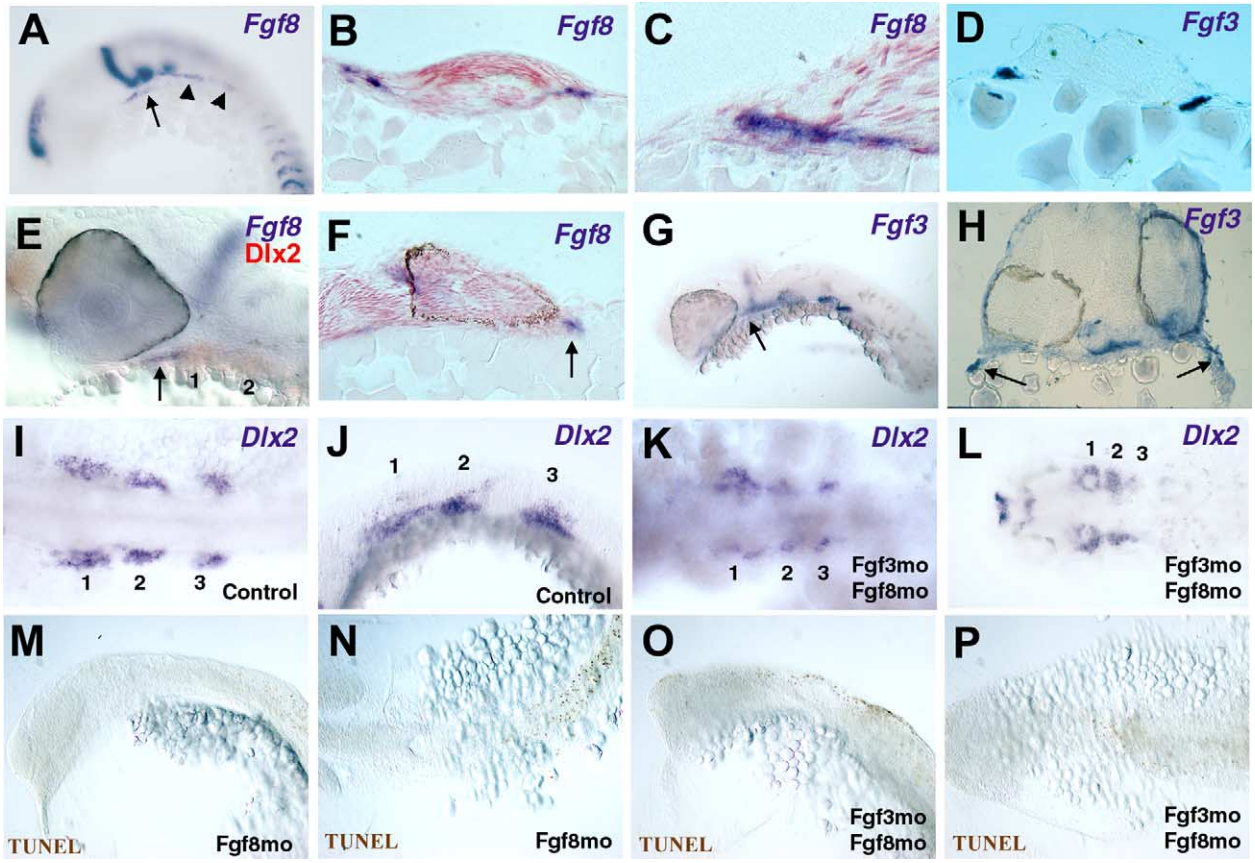

Fig. 6. Endodermal expression of *Fgf8* during early crest migration and coexpression with *Fgf3* in endoderm associated with the jaw. (A) At 16 hpf, *Fgf8* transcripts are detected in first pouch endoderm (arrow) and endoderm (arrowheads) associated with posterior arches. (B, C) Transverse sections through an embryo at 16 hpf showing *Fgf8* expression in endoderm associated with posterior arches (B; region indicated by arrowheads in A) and with anterior arches (C; high power view of one side of a section from the region indicated by arrow in A). (D) Transverse section through the second pharyngeal pouch of a 21-hpf embryo shows *Fgf3* expressed by pharyngeal endoderm. (E–H) At 30 hpf, both *Fgf8* and *Fgf3* are expressed by endoderm associated with the developing jaw. (E) In situ hybridisation for *Fgf8* (blue) and *Dlx2* (orange) transcripts at 30 hpf. *Fgf8* (arrow) is expressed in cells immediately anterior to the first arch (arches 1 and 2 are indicated). (F) Oblique section through the forebrain, eye, and anterior arch region of an embryo at 30 hpf showing Fgf8 transcripts in endoderm (arrow). (G) Fgf3 is expressed in endoderm associated with anterior arches (arrow). (H). Transverse section through a 30-hpf embryo showing Fgf3 expression in anterior endoderm. (I–L) *Dlx2*-positive neural crest cells are still present in embryos at 21 and 30 hpf following inhibition of Fgf3 and Fgf8 translation with morpholinos. The three neural crest cell populations are numbered. In situ hybridisation for *Dlx2* transcripts in embryos at 21 hpf injected with a control morpholino (I, dorsal view; J, lateral view). Three *Dlx2*-positive neural crest populations are detected at 21 hpf following inhibition of both Fgf3mo and Fgf8mo (K; dorsal view); however, at 30 hpf, expression is reduced in the posterior population (L; dorsal view). (M–P) Cell death is not detected in migrating crest streams at 20 hpf following inhibition of either Fgf8 alone (M, N) or both Fgf3 and Fgf8 (O, P). (M, O) Lateral views and (N, P) dorsal views.

adjacent to the isthmus do not (this study; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). These data are supported by studies in *Xenopus* which show Fgf-dependent Erk activity in pharyngeal arch mesenchyme (Christen and Slack, 1999). The period of sensitivity of chondrogenesis in the zebrafish to pharmacological inhibition of FGFR activity corresponds with the period of *Fgf3* and *Fgf8* expression in pharyngeal arch endoderm and of Fgf-dependent *Erm* and *Pea3* expression in migrating neural crest. In addition, injection of Fgf3mo into older embryos such that Fgf3 expression is inhibited in mesenchyme and endoderm, but not neuroepithelium or neural crest, results in reduction or loss of posterior arch cartilage (David et al., 2002). The continued expression of *Fgf3* mRNA following inhibition of Fgf3 and Fgf8, either alone or in combination, indicates that pouch endoderm itself is still present in such embryos (unpublished observations). In the case of Fgf3 inhibition, this is further supported by continued expression of the pouch endoderm markers, *axl/foxA2* and zn5 antigen (David et al., 2002). This, together with expression of Fgf-dependent transcription factors, suggests that Fgf most likely acts directly upon the neural crest cells themselves.

Cartilage formation in arches 3–6 clearly requires Fgf3 function alone, while both Fgf3 and Fgf8 are required together for first, second, and seventh arch cartilage. Both Fgf3 and Fgf8 are expressed in pharyngeal endoderm at the onset of neural crest migration and are most likely to signal to crest at this stage. It is interesting to note that, despite the transient expression of Fgf8 in endoderm associated with arches 3–6, Fgf8 function is not required for cartilage for-

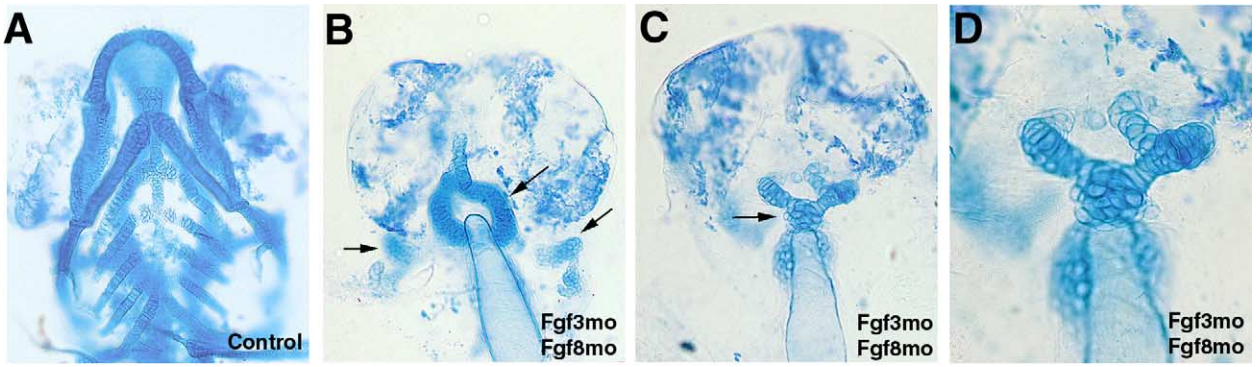

Fig. 7. Fgf3 and Fgf8 are required together for formation of cartilage in the head. (A–D) Ventral views showing near-complete absence of pharyngeal and neurocranial cartilages in 5-dpf embryos in which both Fgf3 and Fgf8 are inhibited. (A) Ventral view of embryo injected with both control morpholinos. (B, C) Embryos in which both Fgf3 and Fgf8 have been inhibited show loss of most cartilage from the head; residual cartilage elements are indicated by arrows. (D) Higher magnification image of (C).

mation in these arches. Fgf8 and Fgf3 are also expressed in pharyngeal endoderm at later developmental stages and hence may also signal to crest cells once they are established within the arches.

In addition to the absence of pharyngeal cartilage in embryos lacking both Fgf3 and Fgf8, most of the neurocranial cartilage is also missing. The source of Fgf3 and Fgf8 signals regulating formation of neurocranial cartilage is unlikely to be pharyngeal endoderm. A more likely candidate is the ventral forebrain, where both Fgf3 and Fgf8 are expressed during the period of neural crest migration and sensitivity to Fgf inhibitors (Walshe and Mason, 2003). A nonendodermal source of Fgf would be consistent with the phenotypes of zebrafish mutants, *Mixer/Bonnie and Clyde (bon)* and *Casanova (cas)*, which lack pharyngeal endoderm and consequently pharyngeal cartilages, but still develop a neurocranial cartilage (David et al., 2002). It is also noteworthy that the identification of mutated genes in zebrafish strains with defective head cartilage formation (Neuhauss et al., 1996; Piotrowski et al., 1996; Schilling et al., 1996a) suggests possible involvement in Fgf-regulated pathways. For example, defects in *Sox* genes, which are known regulators of Fgf expression and also nuclear effectors of Fgf signalling, are responsible for both the *cas* and *jellyfish (jef)* mutations (Dickmeis et al., 2001; Yan et al., 2002). In addition, the *jekyll* mutation is in UDP–glucose dehydrogenase, an enzyme essential for the biosynthesis of heparan sulphate an essential cofactor in Fgf signalling (Walsh and Stainier, 2001).

Studies in other vertebrate classes have not implicated Fgf3 in cranial cartilage development, and defects in posterior arch derivatives were not reported for the *Fgf3*-null mouse (Mansour et al., 1993). However, detailed analysis of pharyngeal cartilages has not been reported for that mutant, and it would be of interest to examine the derivatives of posterior arches (posterior roof of the basihyoid, urohyal, and epi-/ceratobranchials) for defects. By contrast, Fgf8 is clearly required for normal development of pharyngeal arches in the mouse embryo. Introduction of ectopic Fgf8 protein or inhibition of Fgf signalling modulates polarity in murine mandibular explants in vitro (Tucker et al., 1999a, b), while conditional inactivation of *Fgf8* in mouse first arch ectoderm causes similar changes in first arch patterning with subsequent reduction or loss of skeletal elements (Trumpp et al., 1999). Moreover, studies in transgenic mice have implicated Fgf8 in other aspects of arch development, including formation of the forth pharyngeal arch arteries (Abu-Issa et al., 2002; Frank et al., 2002; Vitelli et al., 2002), and studies in chick implicate Fgf8 in frontonasal development (Schneider et al., 2001).

The absolute requirement for Fgf3 and Fgf8 in all pharyngeal and most neurocranial cartilage development in the zebrafish embryo prompts the question of whether other neural crest derivatives also have the same type of requirement for Fgfs. Our analyses of *Sox10* expression in migrating nonectomesenchymal neural crest and formation of trigeminal ganglia, which are partly of neural crest origin, in morpholino-injected embryos indicate that noncartilage-forming neural crest cells express characteristic genes and differentiate normally in the absence of Fgf3 and Fgf8. Although inexhaustive, these data imply that only the cartilage-forming component of the cranial neural crest requires Fgf3 and Fgf8 to differentiate.

In in vitro assays, Fgfs have been variously reported to promote neural crest survival, proliferation, differentiation, and chemotaxis (see e.g., Kalcheim, 1989; Kubota and Ito, 2000; Murphy et al., 1994; Sarkar et al., 2001). By contrast to *cas* mutants, which lack endoderm and show appreciable death of pharyngeal neural crest at 24 and 30 hpf (David et al., 2002), embryos lacking Fgf3 and Fgf8, either alone or in combination, did not show marked increases in crest cell death at 20, 25, 30, or 48 hpf (this study). Cell division was likewise not appreciably affected. The most parsimonious interpretation of our data is that Fgf signalling is required for neural crest to differentiate into cartilage. This is consistent with the results of grafting studies of neural crest with or without associated endoderm and extirpations of mandibular endoderm, which indicated that first arch neural

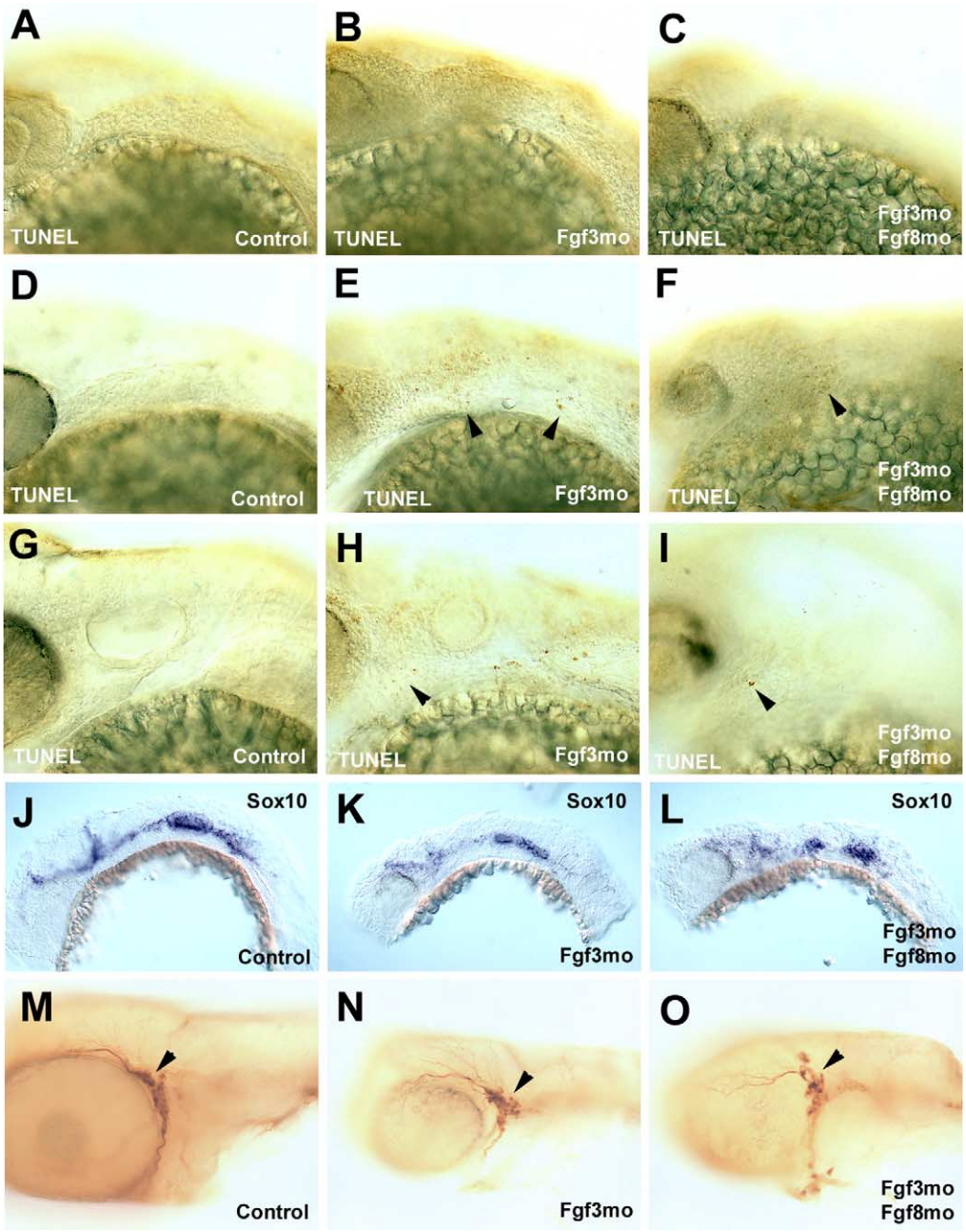

Fig. 8. Effects of inhibition of Fgf3 or Fgf3 and Fgf8 on cranial neural crest cells. (A–I) Injection of Fgf3mo either singly or in combination with Fgf8mo causes little change in cell death in pharyngula stage embryos. Dead cells are extremely rare in the arch regions of embryos injected with control morpholinos at 25 (A), 30 (D), and 48 hpf (G). Injection of Fgf3mo or both Fgf3mo and Fgf8mo results in very little cell death in the arch region at 25 hpf (B, C), while more dead cells (arrowheads) are detected at 30 (E, F) and 48 hpf (H I). (J–O) Fgf3 and Fgf8 are not essential for nonectomesenchymal neural crest cells to express *Sox10* or for formation of the trigeminal ganglion. (J–L) Neural crest cells which do not form cartilage express Sox10 in 24 hpf embryos injected with either control morpholinos (J), Fgf3mo (K), or both Fgf3mo and Fgf8mo (L). (M–O) Formation of the trigeminal ganglion (arrowheads) is unimpeded in embryos injected with either control morpholinos (M), Fgf3mo (N), or both Fgf3mo and Fgf8mo (O).

crest can only differentiate into cartilage in the presence of stomodeal or pharyngeal endoderm. It is noteworthy that midgut and hindgut endoderm, which have not been shown to express Fgfs at early stages of development, were not competent to stimulate cartilage differentiation (Graveson and Armstrong, 1987; Hall, 1980; Seufert and Hall, 1990). In addition, studies of avian cranial neural crest cells in vitro have shown that either stimulation by Fgf or introduction of activated FGF receptors can act directly to promote cartilage formation (Petiot et al., 2002; Sarkar et al., 2001). Taken together, these and our data suggest that endodermal Fgf signalling most likely functions as either an instructive or permissive signal for cartilage differentiation by pharyngeal neural crest cells.

Acknowledgments

We thank Vicki Prince, Robert Kelsh, Frederic Rosa, Herbert Steinbeisser, Marc Ekker, Marina Mione, and Steve Wilson for probes, and Anthony Graham and Imelda McGonnell for suggestions and discussion of the manuscript. This work was supported by the MRC, BBSRC, and Wellcome Trust.

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