# Functional Analyses of FGF during Pharyngeal Cartilage Development in Flounder (Paralichthys olivaceus) Embryo

Authors: Suzuki, Tohru, and Kurokawa, Tadahide

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# Functional Analyses of FGF during Pharyngeal Cartilage Development in Flounder (*Paralichthys olivaceus*) Embryo

Tohru Suzuki* and Tadahide Kurokawa

*Metabolism Section, National Research Institute of Aquaculture, Nansei, Mie 517-05, Japan*

**ABSTRACT**—As the first step to understand the mechanism of pharyngeal cartilage development in the embryo of flounder, *Paralichthys olivaceus*, we studied the process of cartilage formation in the pharynx histologically, then analyzed the role of fibroblast growth factor (FGF) during the process. At hatching (56-62 hr post-fertilization), each mandible, hyoid and gill cartilage existed as a pair of blastemas composed of prechondroblasts. Paired blastemas fused at medial line at 1.5 days post-hatching, forming primordia. Labeling with 5-bromodeoxyuridine indicated that proliferation of prechondroblasts was highly active at this stage. Deposition of cartilage matrix began from the mandible and hyoid primordia at 2.5 days post-hatching, and at 4.0 days the basic patterning of the pharyngeal cartilage was complete.

Immunohistochemical analyses with an antiserum against 22.5 kD FGF (anti-22.5 kD FGF) isolated from swimbladder of red seabream, *Pagrus major*, revealed that the surface ectoderm, gut, lateral line blastema and otic vesicle show FGF-like immunoreactivity. Immunoreactivity signals persisted through the stages when the proliferation of prechondroblasts was active. In order to estimate possible function of 22.5 kD FGF, the blocking effect of anti-22.5 kD FGF towards pharyngeal cartilage formation was assayed using cranial explants dissected from hatched embryos. When incubated with anti-22.5 kD FGF, cartilaginous nodules formed bilaterally at the position of the original blastemas, while in the control treatment, incubation with normal rabbit IgG, cartilages connecting the two lateral sides were formed. Thus, development of pharyngeal cartilage was severely curtailed in the presence of anti-22.5 kD FGF. These results suggest the possibility that 22.5 kD FGF is produced in the pharynx and that it takes part in the regulation of pharyngeal cartilage development, possibly as a signal to stimulate the growth of prechondroblasts.

## INTRODUCTION

In teleost fish, seven pharyngeal arches appear in the pharynx at the late somite stage, then form jaw, hyoid and five gill skeletons that function in feeding and breathing. The mesenchymal population of cells in the pharyngeal arches including cartilage, bone and connective tissue differentiate from cranial neural crest cells (Eisen and Weston, 1993; Sadaghiani and Vielkind, 1989, 1990; Schilling and Kimmel, 1994). These neural crest cells originate from dorso-lateral side of neural keel and migrate under the ectoderm into the pharyngeal region, and finally enter into the pharyngeal arches which are formed by the surface ectoderm and gut endoderm (Sadaghiani and Vielkind, 1989, 1990). The migration pathway and cell fates of cranial neural crest cells are common to those of higher vertebrates. In the embryo of zebrafish, *Danio rerio*, the eventual fates of the cranial neural crest cells, including cartilage and connective tissue lineages, is suggested to be specified before migration (Schilling and Kimmel, 1994). Furthermore, in swordtail, *Xiphophorus helleri*, the cranial neural crest cells that form skeletal elements are reported to have been segregated from connective tissue during migration (Sadaghiani and Vielkind, 1989, 1990). Thus, when compared with chick embryo in which cell lineage is specified during the migration process (Baroffio *et al.*, 1991), the fate specification of the cranial neural crest cells of teleosts seems to occur at an earlier developmental stage.

The cartilage precursor cells originating from cranial neural crest cells become condensed in the pharyngeal arches, forming cell aggregates called blastemas. The cells in the blastema are termed prechondroblasts which are joined to each other by desmosomes and lack extracellular matrix. The prechondroblasts then become chondroblasts, which start secreting a fine fibrillar matrix. Cartilage tissue of this stage is called primordium (Huysseune and Sire, 1992). The primordia become visible as defined protrusions under Nomarski microscopy (Westerfield, 1989). In differentiated cartilage, chondrocytes exist in typical hyaline cartilage matrix. Thus, the differentiation of cartilage of the pharyngeal arch is classified into blastema, primordium and cartilage stages in relation to cell maturation state (Huysseune and Sire, 1992). Due to increase of the cell number and volume and to production of extracellular matrix, cartilage tissue rapidly increase in size through these stages (Huysseune *et al.*, 1988).

* To whom correspondence should be addressed.

Various attractive steps thus exist in pharyngeal cartilage development, such as segmentation, migration and fate destination of cranial neural crest cells, and cell differentiation and proliferation in pharyngeal arch. In lamprey embryo, contact of migrated neural crest cells between pharyngeal endoderm and ectoderm is indicated to be essential for normal development of pharyngeal arch skeleton (Newth, 1956). Such inductive events between pharyngeal epithelium and mesenchyme have been described in more detail in higher vertebrates. For instance, differentiation of neural crest cells to cartilage and neural crest cell growth depend on contact with pharyngeal endoderm in newt and with ectoderm in chick embryos (Epperlein and Lehmann, 1975; Holtfreter, 1968; Tyler and Hall, 1977; Wedden, 1987). From these data, differentiation of neural crest cells to cartilage and their cell growth is thought to be mediated by signals from the epithelia surrounding the pharyngeal arch (Holtfreter, 1968; Hall, 1987). The molecular background of this signaling mechanism is still unclear in fish as well as higher vertebrates. In the limb formation of chick embryo, the proliferation of cartilage precursor cells has been shown to be stimulated by FGF in the manner of epithelium and mesenchyme interaction (Aono and Ide, 1988; Niswander *et al.*, 1993). Since a FGF was previously purified from swimbladder of red seabream, *Pagrus major*, and monospecific antiserum was prepared (Suzuki *et al.*, 1994), this study was conducted to investigate the possibility that this growth factor is involved in the regulation of cell growth at the pharynx of flounder embryos.

The order Pleuronectiformes to which flounders belong includes commercially important fish being cultured in many countries. Both commercial and public hatcheries produce large numbers of fertilized eggs and there is enough surplus for various molecular analyses. In flounder embryos, pharyngeal cartilage has not yet differentiated at hatching and it develops during the successive 3 to 4 days (Bisbal and Bengtson, 1995). It is easy to observe the pharyngeal cartilage formation. For these reasons, we chose to investigate the developmental system of pharyngeal cartilage using flounder, *Paralichthys olivaceus*, embryo. Furthermore, flounder undergoes metamorphosis in its post-larval stage, when head skeleton and skin color are partially remodeled; the bilateral symmetry of the flounder post larva is changed to a characteristic asymmetry with both eyes on the same side of the head, the ocular side of the body being pigmented and the blind side non-pigmented (Brewster, 1987). This metamorphosis is initiated under the control of thyroid hormone (Inui and Miwa, 1985). So, flounder can also serve as a model to analyze the signal network that controls remodeling of neural crest-derived tissues at metamorphosis.

In this report, the process of pharyngeal cartilage formation was first histologically observed, focusing on the pattern of cell proliferation. Next we immunohistochemically tested if 22.5 kD FGF appears at the pharynx. Since several tissues including gut endoderm and surface ectoderm reacted with anti-22.5 kD FGF, we finally analyzed the blocking effect of the antibody toward *in vitro* pharyngeal cartilage formation to infer the function of FGF during pharyngeal cartilage development.

## MATERIALS AND METHODS

*Embryos*

Forty flounders, *Paralichthys olivaceus*, aged 2 - 4 years, consisting of approximately equal numbers of males and females, were kept in a tank of 6 m diameter and 3 m depth. The tank was supplied with sand-filtered seawater at a rate of 0.5 ton/hr. Fish were fed with pellets developed for flounder (Kyowa Hakko Kogyo, Tokyo, Japan) supplemented with 1% fish oil.

Experiments were done in January when the average water temperature in the tank was 13°C. Flounders spawned at around 10 a.m. everyday. A nylon net (200 μm mesh size) was set at overflow of the tank at 12:00 p.m. Two hours later, embryos trapped in the net were collected. At collection, 100,000 - 300,000 embryos were obtained, and their developmental stage was well synchronized at the 4-cell stage. For further development, the embryos were transferred into 20-liter plastic tanks set at 17°C.

The embryos which hatched at 56 hr post-fertilization were selected and incubated at 17°C for further experiments.

*Anti-22.5 kD FGF*

A rabbit antiserum (anti-22.5 kD FGF) was raised against 22.5 kD FGF which was purified from the swimbladder of a red seabream (*Pagrus major*) in the previous report (Suzuki *et al.*, 1994). The immunoglobulin (Ig) fraction was obtained from the antiserum, using Protein A-Sepharose (Pharmacia, Uppsala, Sweden).

*Western blotting of embryonic extract*

Embryos at 1.5 day-post hatching (2.0 g in total wet weight) which had been kept at -120°C were homogenized with ten volumes of 3.0 M NaCl in 10 mM phosphate buffer, pH 7.5 and centrifuged at 15,000 × g for 30 min. The extract was dialyzed against the 10 mM phosphate buffer overnight and centrifuged. The supernatant was loaded on a column (0.5 × 4 cm) containing heparin-Sepharose CL-6B (Pharmacia), which was then rinsed with 10 mM phosphate-buffered saline (PBS). Retained proteins were eluted with 3.0 M NaCl in 10 mM phosphate buffer. The sample was electrophoresed, blotted onto PVDF membrane and immunologically stained with anti-22.5 kD FGF as described previously (Suzuki *et al.*, 1994).

*Histology and immunohistochemistry*

Embryos were fixed every 0.5 days from hatching (56 hr post-fertilization) in 4% paraformaldehyde in 10 mM phosphate-buffered saline (PBS) at 4°C overnight. They were dehydrated through a graded ethanol series, embedded in paraffin, and sectioned at 5 μm. Some of the sections were stained with hematoxylin and eosin.

Deparaffinized sections were incubated with 10% normal goat serum, then in anti-22.5 kD FGF (1:1,000 dilution with PBS). Rabbit Ig absorbed in the sections were visualized using Histofine SAB (R) Kit (Nichirei, Tokyo, Japan), according to the manufacturer's instructions. In the control staining, the serum of pre-immunized rabbit was used.

*Cartilage staining*

Embryos, fixed as described above, were soaked in 0.1% Alcian blue 8GX dissolved in ethanol/HCl mixture (respectively, 70% and 1% in water) for 20 min, washed in the same mixture, dehydrated through a graded ethanol series, and cleared in Lemosol (Wako Pure Chemical, Osaka, Japan). Micrographs were taken using an Olympus IMT reverse-microscope.

*BrdU-labeling*

At 0.5-, 1.5- and 2.5 day post-hatching, fifty embryos were

incubated in 10 ml of 5-bromodeoxyuridine (BrdU) solution (2 mg/ml in 0.4 μm-filtered seawater) for 1 hr at 17°C. They were washed in filtered seawater, fixed in 4% paraformaldehyde (PBS) overnight, dehydrated, and 5 μm-sectioned in paraffin. BrdU-labeled nuclei were visualized by indirect immunofluorescence using anti-BrdU monoclonal antibody and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG as described by Awaji and Suzuki (1995).

*Cartilage formation assay*

Earle's minimum essential medium (MEM; Flow Laboratories, Irvine, Scotland) supplemented with 1% penicillin-streptomycin mixture (Sigma, St Louise, MO, USA) was put into wells (400 μl/each) of a 6-well plastic plate (Falcon, Lincoln, NJ, USA). Either anti-22.5 kD FGF IgG or normal IgG was added to make a final concentration of 1.0 μg/ml. Collagen filter-cups (Costar Transwell-collagen 24.5 mm in diameter, Cambridge, MA, USA) were hung in wells.

Newly hatched embryos were dissected at the level of caudal to otic vesicles, and the anterior explants were collected. Twenty explants were put on the collagen membrane of each filter cup, and then incubated for 4 days at 17°C. After incubation, they were stained with Alcian blue.

## RESULTS

*Cartilage components in newly hatched embryos*

Flounder embryos hatched between 56 and 60 hr post-fertilization when incubated at 17°C. At the time of hatching, the beating heart was clearly observed. Neither the pectoral fin bud nor eye pigment could be detected with Nomarski microscopy (Fig. 1A). On the ventral side of the body, pharyngeal arches, collectively formed a tissue ridge, were seen as a protrusion between the eye and the posterior side of the otic vesicle, expanding slightly laterally over the yolk sac. The antero-posterior length of the protrusion of pharynx was approximately 200 μm. Alcian blue did not yet stain the cartilage matrix in the pharynx at this stage.

The structure of pharyngeal cartilage components in newly hatched embryos was microscopically observed using both transverse and horizontal serial sections (Fig. 1B-D), and schematically reconstructed (Fig. 2). Mandible, hyoid and gill cartilage appeared in the pharynx as a pair of blastemas (Figs.

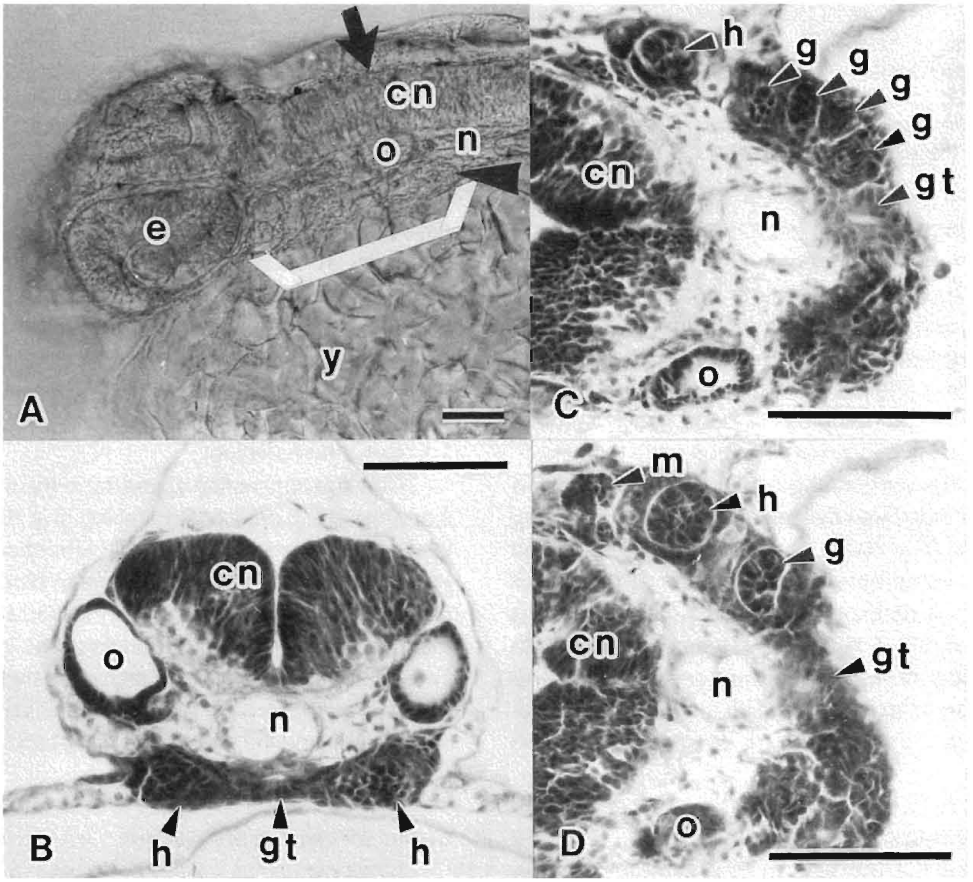


Fig. 1. Pharynx of flounder embryo at hatching period. A, Side view of anterior part of embryo (Nomarski). Protrusion of pharynx including cartilage components, marked by a parenthesis, exists between eye and posterior side of optic vesicles. Arrow and arrowhead indicate the position of sections in B and C-D, respectively. B, Transverse section through otic vesicles. Paired blastemas of hyoid cartilage, composed of prechondroblasts, exist on the both sides of gut. C and D, Sections through the ventral side of the otic vesicles. Section in D located just below that in C. Since sections are slightly inclined from horizontal position, otic vesicle is visible on the left side of the body, and blastemas of mandible, hyoid and gill cartilages (1st - 4th gills in C, only 1st gill in D) are visible on the right side. Scale bars indicate 50 μm. cn, central nervous system; e, eye; g, gill cartilage blastema; gt, gut; h, hyoid cartilage blastema; m, mandible cartilage blastema; n, notochord; o, otic vesicle; y, yolk.

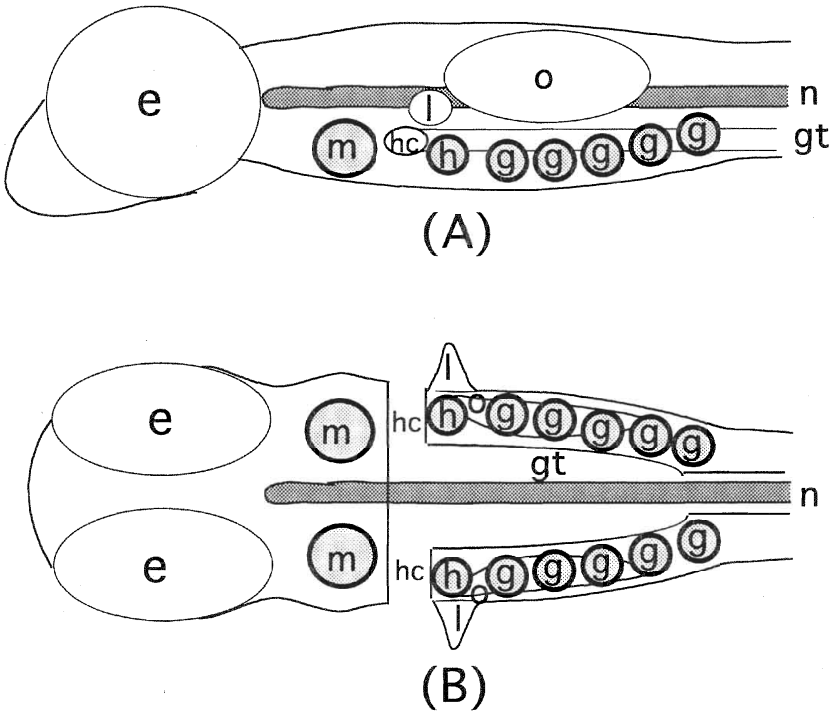


Fig. 2. Schematic arrangement of cartilage components in flounder embryo at hatching period. A, Lateral view. B, Ventral view. Paired blastemas of mandible, hyoid and gill cartilages line in antero-posterior direction. Gut ends at posterior side of mandible blastemas, and bilaterally open via hyomandibular cleft. e, eye; g, gill cartilage blastema; gt, gut; h, hyoid cartilage blastema; hc, hyomandibular cleft; l, lateral line blastema; m, mandible cartilage blastema; n, notochord.

1B-D and 2). The blastemas of the mandibular cartilage were located caudal to the eye. Further caudally, the blastemas of hyoid and gill cartilages followed on both sides of the gut. The gut anteriorly ended at the posterior edge of mandible blastemas, and bilaterally opened to the exterior via hyomandibular clefts (Fig. 2). The otic vesicle was located dorsal to the hyoid and gill blastemas. A lateral line primordium existed anteriorly to the otic vesicle.

In the transverse section, the cartilage blastemas were horizontally flattened and well-delineated from the surrounding mesenchyme tissue (Fig. 1B-D). According to Huysseune and Sire (1992), who described the ultrastructural feature of pharyngeal cartilage development in cichlid *Hemichromis bimaculatus*, these cells of cartilage blastemas correspond to the state of prechondroblasts. In the case of hyoid, the blastema had a maximum diameter of 24 μm in the horizontal sections and was composed of approximately 60 prechondroblasts.

*Accumulation of cartilage matrix at pharynx*

To investigate the process of pharyngeal cartilage formation, embryos were stained with Alcian blue at every 0.5 days after hatching. At 0.5 days post-hatching, the external feature of pharynx did not change from the hatching period, and the protrusion of pharynx including cartilage blastemas could not be seen from the ventral side due to the presence of the yolk sac (Fig. 3A). By 1.0 day post-hatching, mandible had become visible as an arch-like structure (Fig. 3B). At 1.5 days post-hatching, mandible and hyoid had formed cartilage primordia which were seen as V-shaped protrusions which extended from the anteroventral side of otic vesicles to the level of the eye center (Fig. 3C). At this time, Alcian blue-positive substance was not yet detected in the pharynx. These results indicate that paired cartilage blastemas grew and fused at the medial line to form cartilage primordia during the first 1.5 days after hatching.

Cartilage matrix first appeared in the mandible and hyoid primordia at 2.5 days post-hatching (Fig. 3D). Then, 0.5 days later, matrix appeared in the gill primordia. At this stage, the affinity of cartilage matrix with Alcian blue was low. Typical cartilage matrix with strong affinity for Alcian blue appeared in the mandible and hyoid primordia at the 3rd day, and in the 1st and 2nd gill bars at 3.5th day when the mouth opened. At 4.0 days post-hatching, matrix became visible in the 3rd and 4th gill bars, and the basic pattern of pharyngeal cartilage was completed (Fig. 3E).

*Cell proliferation in the pharynx*

Since blastemas initially consisting of a small number of cells grow to a cartilage, a rapid rate of proliferation was predicted in precursor cells at some period of development. In order to determine that period, embryos were incubated with BrdU for 1 hr at 0.5-, 1.5-, 2.5 and 4.0 day post-hatching, and BrdU-labeled nuclei were visualized by indirect immunofluorescence. In 0.5- and 1.5-day post-hatching embryos at blastema and early primordium stages,

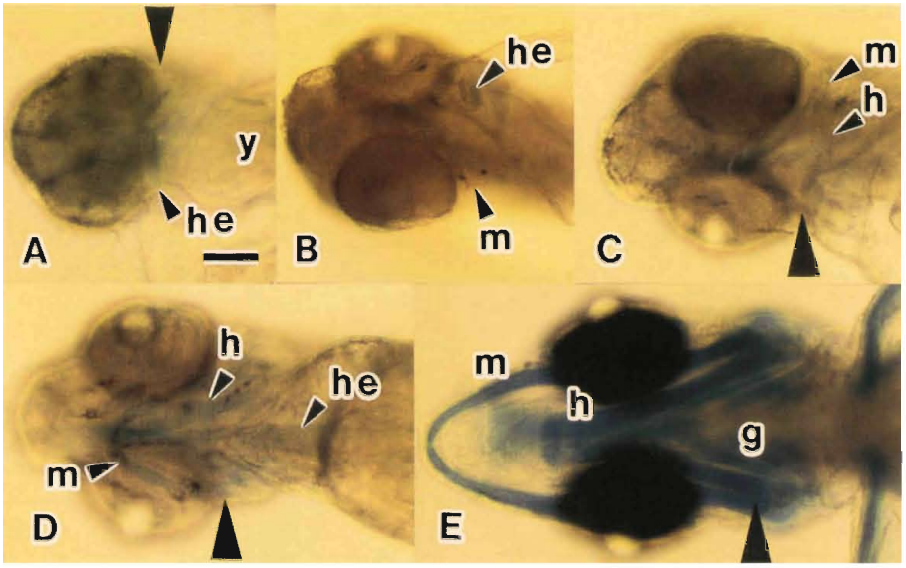


Fig. 3. Process of cartilage formation at pharynx of flounder embryos. The embryos were stained with Alcian blue to visualize cartilage matrix. A, 0.5 day post-hatching. The protrusion of pharynx including cartilage blastemas exists behind yolk sac. Arrowhead indicates the position of section in Figs. 4A-B and 6B. B, 1.0 day post-hatching. Mandible is visible as a small arch. C, 1.5 day post-hatching. Cartilage primordia of mandible and hyoid can be seen as V-shaped structures anteriorly extending from the ventral side of otic vesicles toward median line. Alcian blue-positive matrix was not yet deposited in the primordia. Arrowhead indicate the position of sections in Figs. 4C-D and 6C. D, 2.5 day post-hatching. Cartilage matrix weakly stained by Alcian blue appeared in mandible and hyoid primordium. Arrowhead indicates the position of section in Fig. 4E-F. E, 4.0 day post-hatching. Typical cartilage matrix stained densely with Alcian blue can be seen in mandible, hyoid and gill bars. Arrowhead indicates the position of section in Fig. 6E. Scale bar indicates 100 μm. g, gill; h, hyoid; he, heart; m, mandible; y, yolk sac.

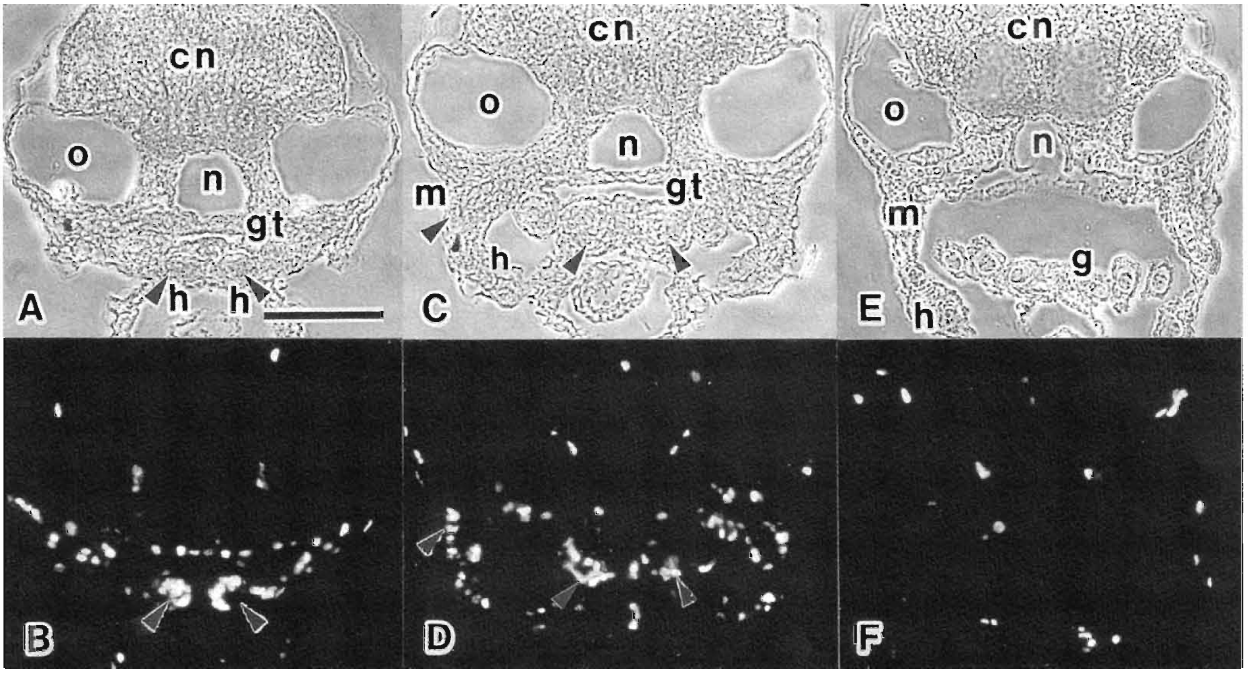


Fig. 4. Cell proliferation at pharynx of flounder embryo. Embryos were incubated with BrdU for 1 hr, then fixed. Labeled nuclei with BrdU were visualized by immunofluorescence (B, D and F). A, C and E are phase contrast micrography of the same sections as B, D and F, respectively. Position of each section is indicated in Fig. 3A, C and D. A-B, 0.5 day post-hatching. Prechondroblasts of hyoid blastemas were mostly labeled (arrowheaded). Upper epithelial cells of gut were also well labeled. C-D, 1.5 day post-hatching. Prechondroblasts of mandible and gill primordia were labeled (arrowheaded). E-F, 2.5 day post-hatching. The cell growth at pharynx had been inactivated. Scale bar indicates 50 μm. cn, central nervous system; g, gill; gt, gut; h, hyoid; m, mandible; n, notochord; o, otic vesicle.

respectively, labeled nuclei were more abundant in the pharyngeal region than in the central nervous system (Fig. 4A -D). Nuclei of prechondroblasts were labeled with BrdU at a particularly high ratio. To a lesser degree, gut epithelial cells were labeled. In 2.5-day post-hatching embryos in which cartilage matrix appeared in the primordia, BrdU-labeled cells

became scarce in the pharyngeal region (Fig. 4E, F). At 4.0 day-post-hatching, chondrocytes were rarely labeled with BrdU, while the liver and pancreas cells were labeled at a high ratio. The distribution pattern of labeled cells was well synchronized in the ten embryos observed at each stage.

*Localization of 22.5 kD FGF*

From the heparin-adsorptive fraction of flounder embryonic extract, FGF-like immunoreactivity was detected by western blot analysis using anti-22.5 kD FGF (Fig. 5). The molecular mass of the positive band was 22,500, coinciding with the FGF isolated from red seabream swimbladder. So, we applied this antibody to immunohistochemistry to test the pharynx of flounder embryos for the presence of 22.5 kD FGF. Weak FGF-like immunoreactivity could be detected from surface ectoderm of the pharynx during the hatching period (Fig. 6A). At 0.5 days post-hatching, immunoreactivity appeared at the gut epithelium, otic vesicle capsule and lateral line primordium in addition to the surface ectoderm (Fig. 6B). Reactivity from these tissues, excepting otic vesicles signals of which had become weak, reached the peak at 1.5 days post-hatching (Fig. 6C). At this time, the pituitary primordium located just anterior to the notochord also exhibited reactivity (Fig. 6D). By 4 days post-hatching, reactivity had disappeared from the surface ectoderm, gut endoderm and otic vesicle (Fig. 6E). In the control staining using serum from pre-immunized rabbit, pharyngeal tissues did not give any immunoreactivity at any stages.

*Blocking of cartilage formation*

In order to investigate the possibility that FGF would be involved in the regulation of pharyngeal cartilage development, we tested the blocking capability of anti-22.5 kD FGF in the cartilage formation. Cranial explants dissected from newly hatched embryos were cultured on collagen membrane in a serum-free medium to which anti-22.5 kD FGF or normal IgG was added. By the 4th day of culture, most explants in both groups had become flattened either with their ventral or dorsal side facing the membrane, and migrating cells from the explants were observed (Fig. 7A, B). Pigmentation and crystalline development occurred in the eyes. In more than 90% of the explants incubated with normal IgG, mandible and hyoid cartilages were formed connecting between the antero-ventral sides of the otic vesicles (Fig. 7A). Gill cartilages formed in some of the explants. These results indicate that histogenesis continued in the cranial explants even in the serum-free conditions.

In the explants cultured with anti-22.5 kD FGF, one or two cartilage nodules of approximately 30 μm of diameter formed antero-ventrally to the otic vesicle (Fig. 7B). Their position coincided with that of the original mandible and hyoid blastemas. However, more posterior gill arch cartilage never chondrified, indicating that pharyngeal chondrification was severely arrested by anti-22.5 kD FGF.

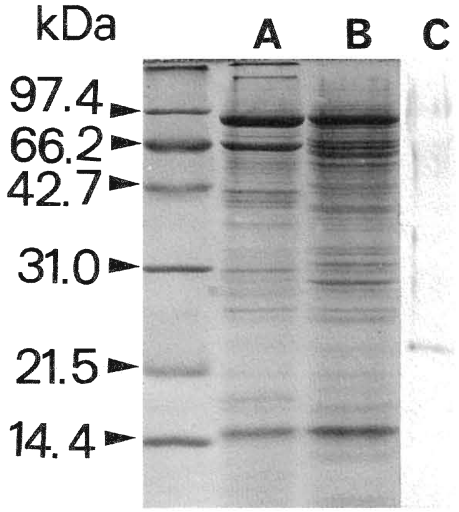


Fig. 5. Detection of FGF-like immunoreactivity from flounder embryonic extract. Heparin-adsorptive fraction prepared from embryonic extract was electrophoresed, blotted onto PVDF membrane and immunostained with anti-22.5 kD FGF. A and B, Coomassie Brilliant Blue staining of electrophoresed embryonic extract and heparin-binding fraction, respectively. C, Western blotting of heparin-binding fraction.

## DISCUSSION

From morphological criteria such as possession of beating heart and undifferentiation of pectoral fin bud and eye pigment, the developmental degree of flounder embryo at the time of hatching is roughly equivalent to the prim-5 stage of the zebrafish (Westerfield, 1993) and to stage 25 of the Medaka (Iwamatsu, 1994). The prim-5 stage is roughly at half of the duration from fertilization to hatching and stage 25 is at the first quarter. Thus, flounder embryos hatch at earlier developmental stage compared with these freshwater teleosts. Comparing the developmental degree of pharyngeal cartilage at hatching, all components including the mandible, hyoid and gills were shown to remain as blastemas in flounder embryos, in contrast to zebrafish and Medaka embryos in which all cartilages are developed prior to hatch (Langille and Hall, 1987; Westerfield, 1989; Schilling and Kimmel, 1994). In addition, cranial explants dissected from newly hatched embryos were found to maintain chondrogenic ability *in vitro* in the serum-free conditions. This *in vitro* system enabled us to analyze the effects of antibodies against secretory proteins on cartilage formation.

In flounder as well as cichlid *H. bimaculatus* embryos (Huysseune and Sire, 1992), blastemas containing a small number of prechondroblasts develop to cartilage with large volume in several days. Such rapid growth of cartilage is attributed to three factors 1) cell proliferation, 2) increase of cell volume and 3) matrix production (Huysseune *et al.*, 1988). To identify the signal that stimulates the proliferation of cartilage precursor cells, it is essential to reveal the stages when their proliferation is active. Our BrdU-labeling test demonstrated that the active period of cell proliferation was the blastema and early primordium stage before deposition of cartilage

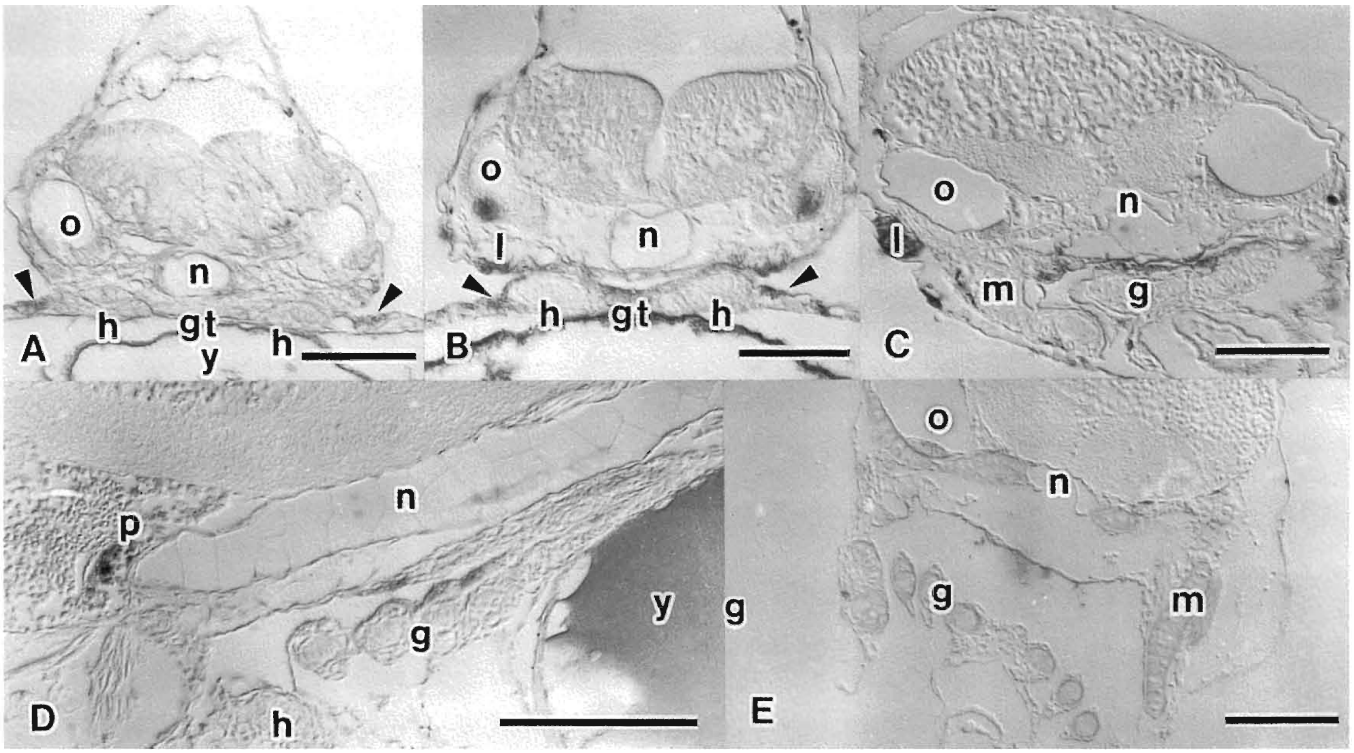


Fig. 6. Immunolocalization of 22.5 kD FGF at pharynx of flounder embryo. Sections were stained by enzyme-labeled immunohistochemistry using anti-22.5 kD FGF. Positions of B, C and E are shown in Fig. 3A, C and E, respectively. A, 0 day post-hatching. Transverse section through otic vesicles. Surface ectoderm (arrowheaded) exhibited weak signals. Yolk sac gave non-specific reaction. B, 0.5 day post-hatching embryo. Gut, otic vesicles, lateral line blastemas and surface ectoderm (arrowheaded) exhibited signals. C-D, 1.5 day post-hatching. Gut, lateral line blastemas gave strong signals, and otic vesicle had weak signals. D is sagittal section through the gut, showing signals of gut epithelium and pituitary primordium. E, 4 day post-hatching. Cartilage matrix was stained with Alcian blue. Signals had disappeared from pharynx. Scale bars indicate 50 μm. g, gill blastema; gt, gut; h, hyoid blastema; l, lateral line blastema; m, mandible; n, notochord; o, otic vesicle; p, pituitary; y, yolk sac.

matrix, and that after matrix appeared in the primordium, cell proliferation became inactivated. From morphometric measurement, it has been demonstrated that the increase of cell volume and the matrix production accounts for most of pharyngeal cartilage growth after differentiation of chondroblasts (Huysseune *et al.*, 1988; Huysseune and Sire, 1992). Taking these data together, the process of cartilage formation in the pharyngeal arch can be summarized as follows; paired blastema grow anteriorly due to the rapid proliferation of prechondroblasts, and are fused at their anterior end to form primordium; during late primordium and cartilage stages, though the cell proliferation is not active, tissue volume increases due the matrix production by chondroblasts and chondrocytes.

The 22.5 kD FGF isolated from the swimbladder of red seabream has an isoelectronic point of 9.2, and exerts growth promoting activity towards fibroblastic and endothelial cells, and also has mesoderm-inducing activity in the animal cap assay using *Xenopus* blastulae, similar to FGFs of higher vertebrates (Suzuki *et al.*, 1994). Western blot analysis indicated that FGF-like immunoreactivity identical in molecular mass with swimbladder-derived FGF is present in the flounder embryo. In immunohistochemistry, immunoreactivity appeared at the surface ectoderm, gut endoderm, otic vesicle and lateral line primordium at the pharynx. The reactivity increased from the hatching period toward the early primordium stage of pharyngeal cartilage, then disappeared with the differentiation of cartilages, indicating that 22.5 kD FGF is present in the pharynx when the proliferation of prechondroblasts is active. From the *in vitro* blocking test, we infer that anti-22.5 kD FGF blocked the proliferation of prechondroblasts, since it severely inhibited the increase in volume from blastema to cartilage. Therefore, we suppose that 22.5 kD FGF is synthesized at the pharynx as one of the signals to stimulate the growth of prechondroblasts during pharyngeal cartilage development of flounder embryo.

In lamprey, newt and chick embryos, it has been demonstrated that signals from gut endoderm and/or surface ectoderm of the pharyngeal arch are necessary both for the differentiation of cranial neural crest cells to cartilage and for their proliferation (Epperlein and Lehmann, 1975; Holtfreter, 1968; Newth, 1956; Tyler and Hall, 1977; Wedden, 1987). In flounder, it is possible that FGF emanating from these epithelia is involved in such signaling mechanism. In the mouse embryo, when cranial neural crest cells are migrating into the pharyngeal arches, FGF-4 is transiently expressed at the foregut endoderm and the surface ectoderm (Niswander and Martin, 1992). So, there is a possibility that FGF is also involved

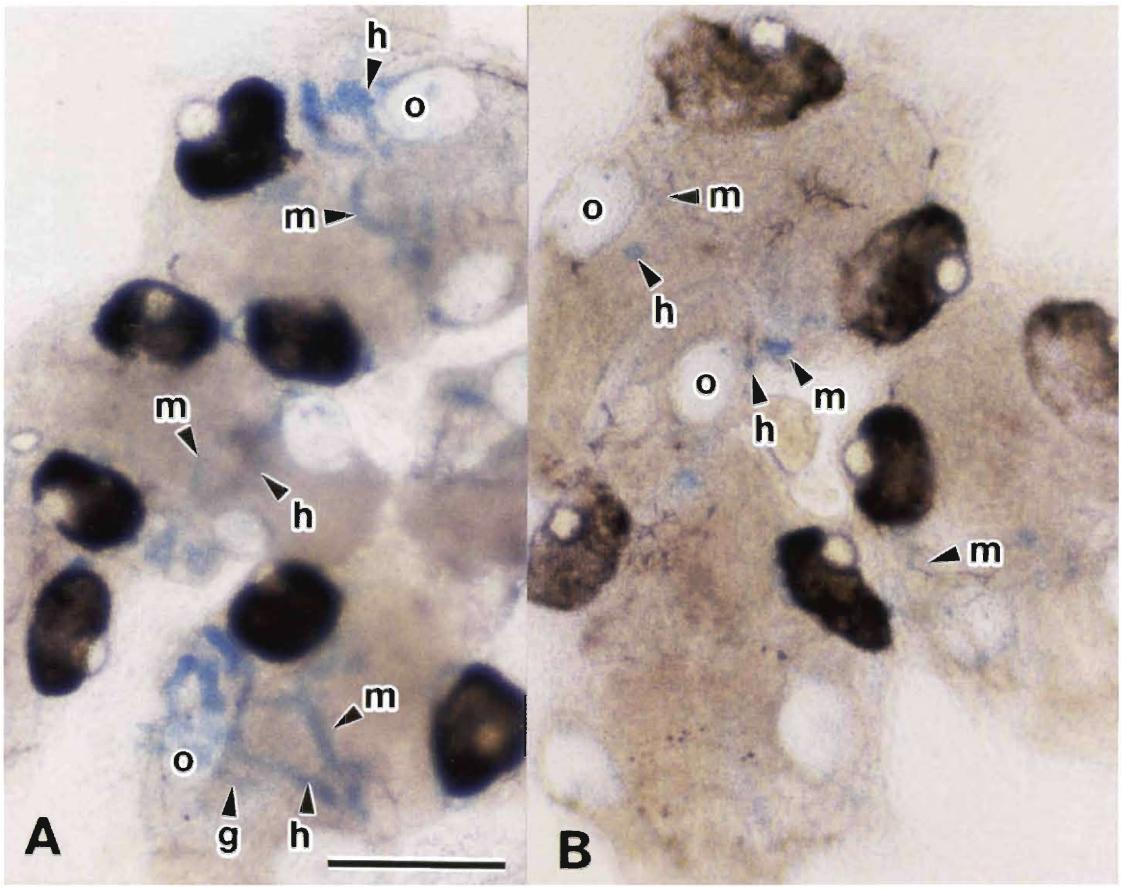


Fig. 7. Blocking effect of anti-22.5 kD FGF toward pharyngeal cartilage formation. Cranial explants obtained from newly hatched flounder embryos were incubated on collagen membrane in serum free condition for 4 days, then stained with Alcian blue. A, explants incubated with normal rabbit IgG (1 μg/ml). In most explants, cartilages developed from mandible and hyoid blastemas were fused. Gill cartilages were formed in some explants. B, explants cultured with anti-22.5 kD FGF (1 μg/ml). Cartilaginous nodules were formed at the position of mandible and hyoid blastemas. Scale bar indicates 200 μm. g, gill cartilage; h, hyoid cartilage; m, mandible cartilage; o, otic vesicle.

in cellular interactions at the pharynx of higher vertebrates.

In the zebrafish embryo, a secretary protein called *Sonic hedgehog* is reported to be expressed at gut endoderm and first gill at the stages when pharyngeal cartilage is developing (Krauss *et al.*, 1993). This protein determines the antero-posterior axis of the skeleton by controlling the expression of *HoxD* genes in chick limb formation (Laufer *et al.*, 1994). In vertebral development, it functions as a signal from notochord and floor plate to differentiate ventral somite to sclerotome (Johnson *et al.*, 1994). Thus, *Sonic hedgehog* is a multifunctional protein with abilities to determine the pattern of skeleton indirectly and to induce differentiation of skeletal elements directly. There is a possibility that *Sonic hedgehog* and FGF work cooperatively in pharyngeal cartilage development; for instance, *Sonic hedgehog* differentiates neural crest cells to cartilage, and FGF stimulates the growth of cartilage precursor cells. To further understand the molecular mechanisms of pharyngeal cartilage development of flounder, we are now analyzing the mRNA expression pattern of several genes including an FGF-4 homologue and *Sonic hedgehog*.

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