Korean J Biol Sci 7: 221-225, 2003

Formation of Sensory Pigment Cells Requires Fibroblast Growth Factor Signaling during Ascidian Embryonic Development

Gil Jung Kim*

Faculty of Marine Bioscience and Technology, College of Life Science, Kangnung National University, Gangneung 210-702, Korea

Key Words:

Pigment cell
FGF signaling
Neural induction
Ascidian embryo

The tadpole larva of the ascidian *Halocynthia roretzi* has two sensory pigment cells in its brain vesicle. To elucidate the temporal requirement for FGF signaling in formation of the pigment cells, embryos were treated with an FGF receptor 1 inhibitor, SU5402, or an MEK inhibitor, U0126 during various embryonic stages. In the present study, it is shown that the embryos treated with SU5402 from the 16-cell stage to the early gastrula stage do not form pigment cells, whereas those treated after the early gastrula stage form pigment cells. In pigment cell formation, embryos suddenly exhibited the sensitivity to SU5402 only for 1 h at the neural plate stage (~4 h after the beginning of gastrulation). When U0126 treatment was carried out at various stages between the 8-cell and late neurula stages, the embryos scarcely formed pigment cells. Pigment cell formation occurred when the embryos were placed in U0126 at early tailbud stage. These results indicate that FGF signaling is involved in pigment cell formation at two separate processes during ascidian embryogenesis, whereas more prolonged period is required for MEK signaling.

Ascidian embryos develop into tadpole-shaped swimming larvae, which have a relatively small number and few types of cells. The larvae have a remarkable features, such as a muscular tail, a notochord and a dorsal neural tube, and based on this, the ascidians are classfied along with vertebrates in the phylum Chordata (Kowalevsky, 1866; Katz, 1983; Satoh, 1994). The central nervous system (CNS) of the larvae is elaborated from the tubular neural cells and lies dorsally from the head region to tip of the tail (Nishida, 1987; Nicol and Meinertzhagen, 1991). The ascidian larval CNS is divided along the anteroposterior axis into brain vesicle (possibly equivalent to the forebrain and midbrain), neck (midbrain or anterior hindbrain), visceral ganglion (hindbrain) and tail nerve cord (spinal cord) (Wada et al., 1998). The a-line animal blastomeres of the blastula generate the brain vesicle (or sensory vesicle), whereas the remaining CNS is derived from the blastomeres of the A- and b-lines (Nishida, 1987). Formation of the brain vesicle is known to require inductive interaction with vegetal blastomeres as found in vertebrates (Rose, 1939; Nishida, 1991; Okamura et al., 1993). The a-line brain precursors isolated prior to neural induction develop into epidermis.

The antagonism of bone morphogenetic protein (BMP) signaling by *chordin* and *noggin* plays a key role in neural induction of *Xenopus* embryos (Sasai and De Robertis, 1997). In ascidian, overexpression of *HrBMPb* and *Hrchordin* does not affect the CNS formation (Darras and Nishida, 2001). However, fibroblast growth factor (FGF) signaling may play a critical role in the ascidian neural induction (Inazawa et al., 1998; Darras and Nishida, 2001; Hudson and Lemaire, 2001). Treatment with basic FGF can induce the expression of neural-specific markers in the a-line neural tissue precursors isolated from 8-cell embryos, but treatment with transforming growth factor-β, activin, epidermal growth factor, and nerve growth factor has no effect. Upon binding to its receptor, FGF activates a protein kinase transduction pathway, which includes Ras, Raf, mitogen-activated protein kinase kinase (MAPKK/MEK), extracellular signal-regulated kinase (MAPK/ERK) through a receptor tyrosine kinase (RTK) (Szebenyi and Fallon, 1999). The requirement for FGF signaling in ascidian neural induction was also shown by injecting the dominant-negative form of Ras and by treatment with inhibitors of FGF receptor (FGFR) and MEK (Kim and Nishida, 2001). It is likely that the requirement for FGF signaling pathway in neural induction ends at the late 64-cell stage. In ascidian embryos, gastrulation starts around the 110-cell stage. FGF signaling has been implicated in the earliest inductive events of neural tissue before gastrulation of

*Tel: 82-33-640-2415, Fax: 82-33-640-2410
E-mail: gjkim@kangnung.ac.kr

chick embryos (Streit et al., 2000). Thus, FGF signaling appears to play evolutionarily conserved roles in neural induction during ascidian and chick embryogenesis.

A typical ascidian larva has two sensory pigment cells, termed the ocellus and the otolith, in its brain vesicle (Nishida and Satoh, 1989; Nicol and Meinertzhagen, 1991). The anterior otolith is sensitive to gravity, whereas the posterior ocellus responds to light. Each cell contains a single melanocyte. The otolith has a large single spherical melanin granule, while the ocellus contains a number of small-sized melanin granules. These melanocytes can easily be distinguished by their morphology and their specific type of pigmentation. It has been reported that three different steps of cell interactions are required for the specification of pigment cells (Darras and Nishida, 2001). The first step can be replaced by FGF treatment, indicating that general neural induction is required as the initial step. At the gastrula stage, BMP signal induces the neuralized cells in close proximity to adopt a pigment cell fate. The final step in this process is the fate specification of otolith versus ocellus by the antagonism of *HrBMPb/Hrchordin*. This final step is achieved at 21 h after fertilization, the midtailbud stage.

The present study was aimed to investigate the temporal requirement for FGF signaling pathway in pigment cell formation during various embryonic stages. I used two specific pharmaceutical inhibitors of FGF signaling, SU5402 and U0126, to block this pathway, and then examined whether pigment cells are formed in the treated embryos.

Materials and Methods

Embryos

Adults of the ascidian, *Halocynthia roretzi*, were collected in the vicinal sea of the province of Gangneung, Korea during the breeding season. Naturally spawned translucent eggs (280 μm in diameter) were artificially fertilized with a suspension of non-self sperm and raised in Millipore-filtered (pore size 0.45 μm) seawater at 13°C. They developed into gastrulae at 10 h and into early tailbud embryos at 19 h after fertilization. Tadpole larvae hatched after 35 h of development.

Treatment with inhibitors

To inhibit the FGF signaling pathway, embryos were treated with 2 μM SU5402 (Calbiochem, San Diego, USA) for 2 h, or 2 μM U0126 (Promega, Madison, USA) for 20 min (Kim and Nishida, 2001). They were rinsed several times with the filtered seawater and then raised until the hatching stage. SU5402 belongs to a new family of FGF signaling inhibitors that bind specifically to the active sites of FGFR kinase domains (Mohammadi et al., 1997). U0126 is an MEK inhibitor that inhibits both the activation of MEK by Raf and the activation of MAPK by MEK (Favata et al., 1998). It was found that 2 μM SU5402 produced a strong inhibiting effect in notochord and mesenchyme formation in several experiments, whereas concentrations higher than 4 μM affected embryonic cleavages (data not shown). U0126 gave the same phenotype at concentrations ranging from 2 μM to 10 μM without affecting cleavages. Therefore, the concentration of 2 μM was chosen as a working concentration for both inhibitors. The inhibitors were dissolved in dimethylsulfoxide at a concentration of 10 mM and stored at -20°C. The stock solutions were diluted with the filtered seawater to the final concentration just before use.

Microinjection of Ras

Ras protein was microinjected into fertilized eggs as described by Nakatani and Nishida (1997). The dominant-negative form of Ras (Ras^{N17}) and wild-type Ras were solubilized in injection buffer, which consists of buffer A (50 mM HEPES NaOH (pH 7.5), 5 mM $MgCl_2$, 5 mM DTT, 1 mM PMSF) plus 50% glycerol and then mixed with 1 mg/ml Fast Green in water. The final concentration of Ras protein to be injected was approximately 1 mg/ml. The injected volume of Ras solution was approximately 5% of the total volume of eggs.

Results and Discussion

In ascidian embryos, FGF signaling has been proposed to be involved in the specification of pigment cells (Nakatani and Nishida, 1997; Darras and Nishida, 2001; Kim and Nishida, 2001). However, it is still not clear when FGF signaling play crucial roles in pigment cell formation. To precisely determine the temporal requirement for FGF signaling pathway in pigment cell formation, embryos were treated with SU5402 or U0126 at various cleavage stages. Both inhibitors are useful tools for blocking endogenous FGF signaling in the ascidian embryo (Kim and Nishida, 2001). Treatment with SU5402 for 2 h was started every 1 h from the 8-cell stage to the early tailbud stage. Treatment with U0126 for 20 min was carried out at intervals of 1 h during the 8-cell and early tailbud stages. The treated embryos were cultured until the hatching stage and then observed for formation of melanocytes. The results are summarized in Fig. 1.

When embryos were cultured in SU5402 (FGFR inhibitor) at the 8- and early 16-cell stages, they formed pigment cells in most cases (Fig. 1A). In contrast, the resulting larvae scarcely showed sensory pigment cell formation when embryos were treated with SU5402 between the late 16-cell stage and the early gastrula (110-cell) stage (Fig. 2A). Embryos placed in SU5402 from 1 h after the beginning of gastrulation formed pigment cells (Fig. 2C). They showed neural tube formation, and had elongated tails and morphologically identifiable

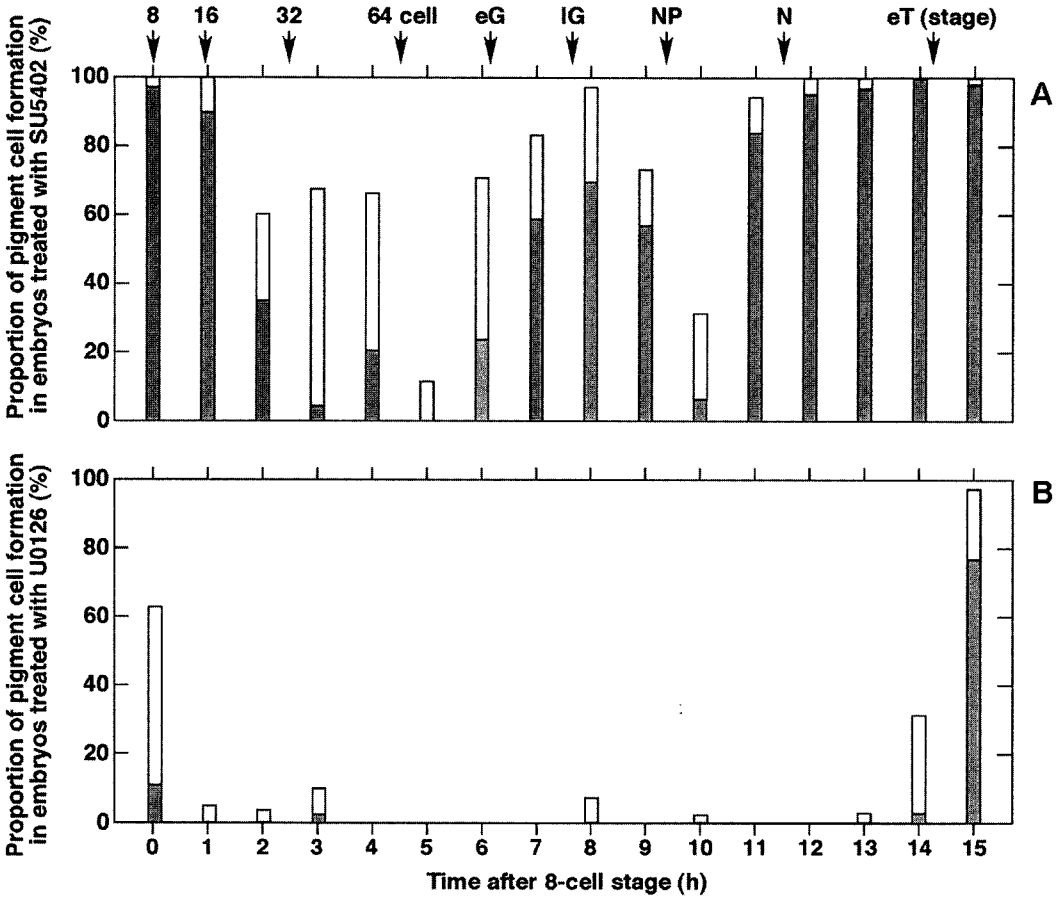

Fig. 1. Results of experiments designed to examine the temporal requirement for FGF signaling pathway in pigment cell formation. Formation of pigment cells was analyzed by observing formation of melanocytes in the resulting larvae. Embryos were treated with SU5402 (A) for 2 h or U0126 (B) for 20 min at the time points (intervals of 1 h) shown along the x-axis between the 8-cell stage and the early tailbud stage. Relative time zero corresponds to the time of the first treatment. Above the x-axis, embryonic stage was indicated. Each gray shaded bar represents the proportion of the specimens with two melanocytes. Each white bar represents the proportion of specimens that had one melanocyte. At least 40 embryos were scored for each time point; eG, early gastrula; lG, late gastrula; NP, neural plate; N, neurula; eT, early tailbud.

brain vesicle. However, when the treatment was started approximately 4 h (termed the neural plate stage) after the beginning of gastrulation, most embryos did not form pigment cells in their brain vesicle (Fig. 2D). SU5402 did not inhibit formation of pigment cells when embryos were placed after the early neurula stage (Fig. 2E). These results indicate that FGF signal is necessary for pigment cell formation at two separate steps during ascidian embryogenesis. It is likely that the first step is achieved from the late 16-cell stage to the early gastrula stage, and the second step is performed at the neural plate stage.

Previous report suggests that FGF signal is required for pigment cell formation from the late 16-cell stage to the 64-cell stage, termed the initial step of general neural induction (Darras and Nishida, 2001). In this study, it is also shown that FGF signal is involved in the specification of pigment cells after neural induction. Recently, the homolog of the vertebrate FGF4/6 and FGF9/20, *Cs-FGF4/6/9*, was isolated from the ascidian *Ciona savignyi* (Imai et al., 2002). The early embryonic expression of *Cs-FGF4/6/9* was transient and the transcript was seen in the cells of endoderm, notochord, muscle, and nerve cord. Differentiation of mesenchyme and notochord cells inhibited when the gene function was suppressed with a specific antisense morpholino oligonucleotide. In contrast, *Cs-FGF4/6/9* does not play a critical role in the specification of the CNS, because the suppression of its function with the morpholino did not reduce expression of the neural tissue-specific genes. However, it remains unclear whether FGF signals other than FGF4/6/9 play an essential role in the formation of the CNS and pigment cells. In neurula, *Cs-FGF4/6/9* was then expressed again in cells of the CNS (Imai et al., 2002). Thus, it can be conceived that the specification of pigment cells is associated with the FGF signal expressed in the CNS. In future studies, cloning of FGF genes in *Halocynthia* embryos and more comprehensive investigation of roles of the FGFs in the embryonic development will be important.

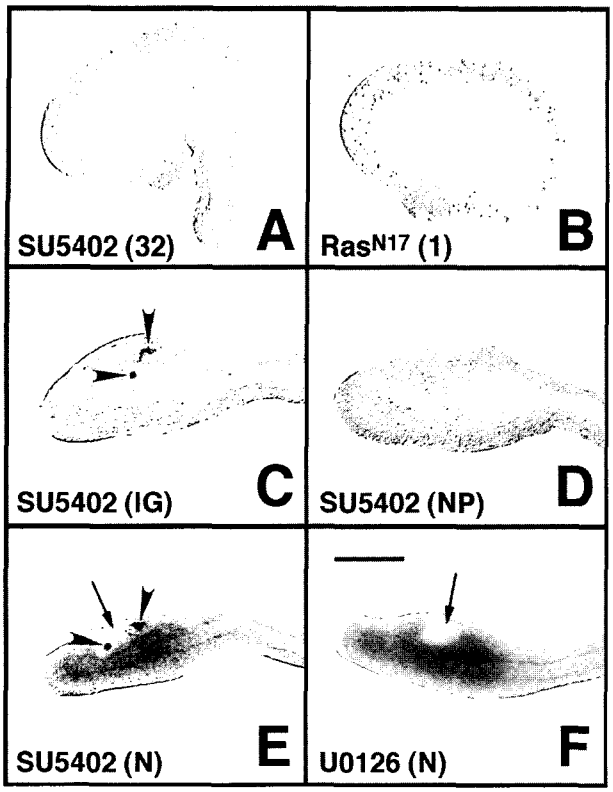

Fig. 2. Larvae just after the hatching stage. Anterior is to the left. A, Larva treated with SU5402 for 2 h from the 32-cell stage. B, Larva injected with Ras^{N17} at the 1-cell stage. C-D, Larvae placed in SU5402 for 2 h at the late gastrula stage (C), or at the neural plate stage (D). To precisely determine pigment cell formation, the larvae were flatted on slideglass (A-D). E-F, Larvae treated with SU5402 (E) for 2 h or U0126 (F) for 20 min from the neurula stage. Arrows indicate the brain vesicle and arrowheads indicate the melanocytes. Scale bar=100 μm.

When embryos were cultured in U0126 (MEK inhibitor) between the 8-cell stage and the late neurula stage, they did not form pigment cells in most cases (Fig. 1B). U0126 strongly suppressed formation of pigment cells at the neural induction and neurula stages. When embryos were treated with U0126 at the neurula stage, the resulting larvae had brain vesicle, but no pigment cells (Fig. 2F). The sensitivity to U0126 suddenly ended at ~19 h after fertilization (early tailbud stage). Embryos placed in U0126 from the early tailbud stage formed pigment cells in most cases (Fig. 1B). As shown in Fig. 2B, it was reconfirmed that embryos injected with dominant negative Ras^{N17} scarcely formed pigment cells (12% of cases; n=17), as reported in previous studies (Nakatani and Nishida, 1997; Kim and Nishida, 2001). Embryos injected with wild-type Ras showed pigment cell formation and normal morphology in 86% of cases (n=14). Therefore, Ras-MEK-MAPK signaling is involved in pigment cell formation up to the early tailbud stage, whereas FGF signal is not necessary after the neural plate stage.

In *Halocynthia* embryos, melanization of the pigment cell precursors is controlled by activity of tyrosinase after 17 h of development (late neurula) (Nishida and Satoh, 1989). The resulting larvae showed pigment cell formation when activation of FGFR was blocked from the late neural plate stage to the early tailbud stage (Figs. 1A, 2E). Thus, it is possible that another RTK, which stimulates Ras-MEK-MAPK signaling, is involved in the specification of sensory pigment cells at these stages. Another possibility is that activity of MEK stimulated by FGF signal remains until the early tailbud stage. Very recently, the spatial and temporal pattern of MAPK activation was shown in the ascidian embryo (Nishida, 2003). Activated MAPK was accumulated in the nuclei of brain precursors from early 32- to 110-cell embryos. In late gastrula and neurula, activation of MAPK was predominantly observed in the brain precursors. This report supports the results of the previous and present studies that FGF and MEK signaling cascade is required for neural induction and pigment cell formation.

Acknowledgements

I thank Dr Hiroki Nishida (Tokyo Institute of Technology) for his support and advice. This work was supported by Korea Research Foundation Grant (KRF-2001-002-D00263).

References

Darras S and Nishida H (2001) The BMP/CHORDIN antagonism controls sensory pigment cell specification and differentiation in the ascidian embryo. *Dev Biol* 236: 271-288.

Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, and Trzaskos JM (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem* 273: 18623-18632.

Hudson C and Lemaire P (2001) Induction of anterior neural fates in the ascidian *Ciona intestinalis*. *Mech Dev* 100: 189-203.

Imai KS, Satoh N, and Satou Y (2002) Early embryonic expression of FGF4/6/9 gene and its role in the induction of mesenchyme and notochord in *Ciona savignyi* embryos. *Development* 129: 1729-1738.

Inazawa T, Okamura Y, and Takahashi K (1998) Basic fibroblast growth factor induction of neuronal ion channel expression in ascidian ectodermal blastomeres. *J Physiol* 511: 347-359.

Katz MJ (1983) Comparative anatomy of the tunicate tadpole, *Ciona intestinalis*. *Biol Bull* 164: 1-27.

Kim GJ and Nishida H (2001) Role of the FGF and MEK signaling pathway in the ascidian embryo. *Dev Growth Differ* 43: 521-533.

Kowalevsky A (1866) Entwicklungsgeschichte der einfachen ascidien. *Mem Acad Imp Sci St Petersbourg* 10: 1-19.

Mohammadi M, McMahon G, Sun L, Tang C, Hirth P, Yeh BK, Hubbard SR, and Schlessinger J (1997) Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. *Science* 276: 955-960.

Nakatani Y and Nishida H (1997) Ras is an essential component for notochord formation during ascidian embryogenesis. *Mech*

Dev 68: 81-89.

Nicol D and Meinertzhagen IA (1991) Cell counts and maps in the larval central nervous system of the ascidian *Ciona intestinalis* (L.). *J Comp Neurol* 309: 415-429.

Nishida H (1987) Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme. III. Up to the tissue restricted stage. *Dev Biol* 121: 526-541.

Nishida H (1991) Induction of brain and sensory pigment cells in the ascidian embryo analyzed by experiments with isolated blastomeres. *Development* 112: 389-395.

Nishida H (2003) Spatio-temporal pattern of MAP kinase activation in embryos of the ascidian *Halocynthia roretzi. Dev Growth Differ* 45: 27-37.

Nishida H and Satoh N (1989) Determination and regulation in the pigment cell lineage of the ascidian embryo. *Dev Biol* 132: 355-367.

Okamura Y, Okado H, and Takahashi K (1993) The ascidian embryo as a prototype of vertebrate neurogenesis. *Bioessays* 15: 723-729.

Rose SM (1939) Embryonic induction in the ascidia. *Biol Bull* 76: 216-232.

Sasai T and De Robertis EM (1997) Ectodermal patterning in vertebrate embryos. *Dev Biol* 182: 5-20.

Satoh N (1994) Developmental Biology of Ascidians. Cambridge University Press, New York, pp 69-131.

Streit A, Berliner AJ, Papanayotou C, Sirulnik A, and Stern C (2000) Initiation of neural induction by FGF signaling before gastrulation. *Nature* 406: 74-78.

Szebenyi G and Fallon JF (1999) Fibroblast growth factors as multifunctional signaling factors. *Int Rev Cytol* 185: 45-106.

Wada H, Saiga H, Satoh N, and Holland (1998) Tripartite organization of the ancestral chordate brain and the antiquity of placodes: Insights from ascidian *Pax-2/5/8*, *Hox* and *Otx* genes. *Development* 125: 1113-1122.

[Received April 26, 2003; accepted June 24, 2003]