Title	金魚の黒色素胞の分化と移動に関する免疫組織化学的研究
Sub Title	Immunocytochemical studies on the differentiation of melanophores and its relation to migratory behaviors in goldfish “Carassius auratus”
Author	秋山, 豊子(Akiyama, Toyoko) 松本, 二郎(Matsumoto, Jiro) 北村, 邦夫(Kitamura, Kunio)
Publisher	慶應義塾大学日吉紀要刊行委員会
Publication year	2006
Jtitle	慶應義塾大学日吉紀要. 自然科学 No.39 (2006.) ,p.1- 19
JaLC DOI	
Abstract	The melanophores of goldfish (Carrassius auratus) are investigatedduring embryogenesis on their differentiation and migratory behaviors fromneural crest (embryonic shield) cells by means of immunocytochemical studiesusing neural crest specific antibody (HNK-1) and melanoblast specific antibody (MEBL-1) . In preliminary assay using cells derived from neural crest explants,neural crest cells under migration unequivocally cross react with HNK-1 andmelanoblasts with MEBL-1 until onset of melanogenesis, respectively. To disclosemelanophore behavior more precisely, cryosectioned embryos and larvae wereassayed and we obtained following results: (1) the HNK-1 positive cells firstappear in the dorsal ridge of the neural tube at stage 20 and then distribute overall sites of both dorsolateral and ventral pathways, (2) the MEBL-1 positive cellsfirst appear among the neural crest derivatives distributing outside of the neuraltube, suggesting the melanization started during migration, (3) huge number ofmelanoblasts/phores migrate trough dorsoventral route in goldfish, different fromavian and mammals. (4) the melanoblasts and melanophores are found closelyat staring point of migration at stage 20 and later, found at more migratedposition, suggesting the presence of a pioneer which develops the routes. Thisstudy clearly shows that melanoblasts/cytes in goldfish migrate through not onlydorsolateral route but also dorsoventral route. The development and theirfunction of large number of pigment cells invaded into almost all internal organsare discussed.
Notes	
Genre	Departmental Bulletin Paper
URL	https://koara.lib.keio.ac.jp/xoonips/modules/xoonips/detail.php?koara_id=AN10079809-20060000-0001

Hiyoshi Review of Natural Science
Keio University No. 39, 1–20 (2006)

Immunocytochemical studies on the differentiation of melanophores and its relation to migratory behaviors in goldfish *Carassius auratus*

Toyoko AKIYAMA, Jiro MATSUMOTO and KUNIO KITAMURA

Summary —— The melanophores of goldfish (*Carrassius auratus*) are investigated during embryogenesis on their differentiation and migratory behaviors from neural crest (embryonic shield) cells by means of immunocytochemical studies using neural crest specific antibody (HNK-1) and melanoblast specific antibody (MEBL-1). In preliminary assay using cells derived from neural crest explants, neural crest cells under migration unequivocally cross react with HNK-1 and melanoblasts with MEBL-1 until onset of melanogenesis, respectively. To disclose melanophore behavior more precisely, cryosectioned embryos and larvae were assayed and we obtained following results: (1) the HNK-1 positive cells first appear in the dorsal ridge of the neural tube at stage 20 and then distribute over all sites of both dorsolateral and ventral pathways, (2) the MEBL-1 positive cells first appear among the neural crest derivatives distributing outside of the neural tube, suggesting the melanization started during migration, (3) huge number of melanoblasts/phores migrate trough dorsoventral route in goldfish, different from avian and mammals. (4) the melanoblasts and melanophores are found closely at staring point of migration at stage 20 and later, found at more migrated position, suggesting the presence of a pioneer which develops the routes. This study clearly shows that melanoblasts/cytes in goldfish migrate through not only dorsolateral route but also dorsoventral route. The development and their function of large number of pigment cells invaded into almost all internal organs are discussed.

Key Words: Pigment cell, HNK-1, Melanoblast-specific antibody (MEBL-1), chromatoblast, migration route

秋山豊子，松本二郎，慶應義塾大学生物学教室，北村邦夫，三菱化学生命科学研究所，発生（連絡先：秋山豊子，〒223-8521 横浜市港北区日吉4-1-1：Dept. of Biology, Keio University., 4-1-1 Hiyoshi, Yokohama, 223-8521, Japan）[received Oct. 21, 2005]

Introduction

Neural crest cells are unique cell group that derived from same neural crest after neurula but have pluripotent function and differentiate into a variety of progenies such as peripheral neurons, glial cells, mesenchymal cells and so on (Weston, 1970; Le Douarin, 1980, 1982; Graham, 2003; Tucker, 2004) during migration from neural crest. Pigment cells in vertebrates are also known to be derived from the neural crest and have been considered to occur in response to a cue from tissue environments after migration from the neural crest. Because of easiness in observing its phenotype; pigmentation, pigment cells have long been one of the most excellent objects for studies on the mechanisms of neural crest differentiation (Kelsh et al., 2000; Quigley & Parichy, 2003). But to analyze the differentiation mechanism of pigment cell before the melanization, we need useful marker to detect unpigmented precursor of pigment cells.

Thus far, the behavior and fate of neural crest derivatives have been intensively studies on avian species either by grafting their tissue pieces labeled with radioisotopes (Weston, 1963) or making chimera between a chick and quail install with distinct built-in markers (Le Douarin, 1980). Monoclonal antibodies against neural crest markers (NAPA-73, HNK-1 and NC-1 etc.) has successfully made it possible to visualize migratory behavior of neural crest derivatives during embryogenesis (Ciment & Weston, 1982, 1985, Tucker et al., 1984; Bronner-Fraser, 1986; Ericson et al., 1989; Newgreen et al., 1990). Also successful availability of new vital dyes has made it possible to trace such migratory cells in living embryos (Serbedzija *et al*., 1989). A series of studies using these new tracing probes have decisively confirmed the migratory pathways of neural derivatives previously reported by Weston (1970) and Le Douarin (1982) : In avian embryogenesis, the derivatives take either the dorsolateral route laid along the ectoderm or the ventral route laid between the somite and the neural tube. These studies have also clearly discriminated the properties of neural crest derivatives under migration and at settlement. Several reviews reported current progress in neural crest motility and migration that extracellular matrix (ECM : Fibronectin, laminin and collagen etc.) accelerate neural crest cells migration and meanwhile certain proteoglycans, agglutinines and protein factors such as Ephrins and their receptors (Ephs) inhibit neural crest cell migration (Grahams, 2003; Hallorans & Berndt, 2003; Coles, et al., 2006). From *in vivo* and *in vitro* studies using avian neural crests, at least 3 types of integrin located on the cell surface of neural crest cells as receptors related to each ligands of ECM. N-cadherin and NCAM also play some roles on cell to cell attachment of neural crest cells in migrating phase. T cadherin and determine

their migratory position of somite whether former or latter part in each somite. The involvement of neuropilin 2/semaphoron 3F signaling in route guidance of trunk neural crest migration is also reported (Halloram & Berndt, 2003; Grammill et al, 2006). These factors are suggested to be related to identification and patterning of the neural crest derivatives.

Serbedzija *et al.* (1989; 1990) have indicated using such new probes that avian pigment cells generally take only the dorsolateral route for their distribution, migrating out from the top of the neural tube as the latest group around the stage 20.

In lower vertebrates, it is known that three to five different types of pigment cells, melanophores, xantho/erythrophores and iridophores, are derived from the neural crest (Obika, 1963; Richards & Bagnara, 1967; Weston, 1970; Bagnara, 1987) and that there are regional differences in the neural crest for the capability of yielding melanophores and xanthophores (Kajishima, 1958). These findings apparently imply that neural crest cells differentiate into pigment cells with determined phenotype through chromatoblasts which still maintain plasticity for making either melanin, or pteridines or reflecting substance. It has been known that HNK-1 antibody does react with a glycoside chain of NCAM produced on cell surface of neural crest derivatives in wide extent of animal embryos (Sadaghiani & Vielkind, 1989; 1990ab; Matsumoto et al, 1989) On the other hand, Kitamura et al. have produced monoclonal antibody (MEBL-1) which recognizes a melanosomes membrane protein molecule as an epitope present in avian melanoblasts (Kitamura et al., 1992). It is expected that combined application of these two antibodies on fish embryos would clarify stepwise differentiation of neural crest cells into melanophores via chromatoblasts and melanoblasts.

The purpose of this study is to clarify (1) the timing by which the neural crest cells of fish origin are subjected to differentiation toward pigment cells with a given phenotype like melanophore, via pluripotent propigment cells (chromatoblasts) and pigment phenotype-committed blast cells (melanoblasts, xantho/erythroblasts and iridoblasts), and (2) possible role of tissue environments in determination of pigment phenotype during migration and settlement.

Materials And Methods

Collection of embryos and larvae

Fertilized eggs of homogeneously orange-colored goldfish *Carassius auratus* were obtained by mating mature adults of commercial origin in the laboratory aquarium or provided by Tokyo Metropolitan Fisheries Experiment Station on Katsushika, Tokyo where

natural spawning was made in an outdoor pond. The eggs were sterilized by exposing briefly to malachite green (Sigma, M6880, St. Louis) at a concentration of 200-300 ppm and then maintained in methylene blue-added dechlorinated tap water at room temperature. The developmental stages were identified according to the nomenclature by Kajishima (1960).

Monolayer culture of neural crest *in vitro*

The neurula (stage 15) embryos were sterilized by brief rinsing in 70% ethanol and their egg capsules were removed by a sharp pointed forceps. The whole embryonic shield was first separated from the yolk sac and then trimmed into three parts of similar size along the orientation perpendicular to the antero-posterior axis in Ca^{++}, Mg^{++}-free phosphate buffered saline (CMF-PBS: 123.2 mM NaCl, 2.4 mM KCl, 7.3 mM Na_2HPO_4, 1.3 mM KH_2PO_4, pH 7.3) with use of a tungsten needle. A rectangular tissue piece dissected out from the central portion of the shield was further trimmed into three parts along the antero-posterior axis. Tissue pieces thus obtained were placed on a poly-L-lysin (Sigma, p-1274, St. Louis) coated glass cover slip, rinsed in Leibovitz 15 medium supplemented with 15% fetal calf serum and cultured at 25℃ after sealing it in a small culture dish. The explants were anchored on a cover slip within an hour and the outgrowth was observed on the next day. Cover slip cultures of neural crest explants were fixed on the first to fourth day of cultivation by the procedure described in the following section for immunofluorescence.

Cryosectioning

The embryos at varying stages from gastrula (stage 20) to hatching (stage 25) and the larvae up to the stage 28 were fixed with 3.5% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 2 hours or overnight at 4℃. The fixation of these embryos were performed after removal of the corion with use of a forceps, using about 10 specimens per one stage. The fixed specimens were washed in phosphate-buffered saline (PBS) for 30 minutes with three exchanges, rinsed in 10% sucrose in PBS for overnight at 4℃ with gentle stirring, and finally transferred to 20% sucrose in PBS. The specimens were frozen in O.C.T.-compound (Tissue Tek Miles Lab. Inc. Elkhart) over dryice-etanol using an aluminum basket and cryosectioned on a Reichert-Jung E microtome. Serial cryosections were attached on 0.02% poly-L-lysine-coated slide glass, every other section being placed alternatively on separate slides to facilitate comparison between labeling by different antibodies.

Immunofluorescence

Cryosectioned specimens were thawed in a room temperature and rinsed in CMF-PBS to remove tissue-mounting compound. The sections were then exposed to one of the monoclonal antibodies, HNK-1 (Becton-Dickinson, No. 7390, Mountain View) and MEBL-1 which was produced by Kitamura *et al.,* (1992). The specimens were then exposed to either the HNK-1 antibody at 50-times dilution in CMF-PBS containing 1% bovine serum albumin (BSA) or the MEBL-1 without any dilution. All the sections thus labeled were then washed in CMF-PBS for 15 minutes with three changes and incubated in biotinylated antibody of the anti-mouse whole immunoglobulins from sheep (Amersham Japan, RPN 1001) at 50-times dilution in CMF-PBS containing BSA at room temperature. Following rinsing in CMF-PBS, these were exposed to fluorescein isothiocyanate (FITC) -conjugated streptavidin (Amersham Japan, RPN 1232) at 100 times dilution in PBS, and washed again in the same way as before. Then the specimens were mounted in glycerin and examined under an epifluorescence microscopy, Nikon Fluophot. Photomicrography was made using an ASA 400.

Results

Reactivity of neural crest cells *in vitro* and *in vivo* and its change during the differentiation

When a small piece of the neural crest (embryonic shield) tissue was dissected out from the neurula embryo (stage 15) and cultured on a glass slip, numerous cells migrated out from the explants. Within 35 hours after cultivation *in vitro*, certain numbers of cells in the outgrowth become pigmented either in faintly brown or pale yellow. And on the third day, when the midcultured part of the neural crest was adopted, the vast majority of the outgrowth was differentiated into melanophores and xanthophores.

Immunocytochemical assay using these cultures under different stages of differentiation disclosed that to the HNK-1 antibody, the explants of the one-day-old cultures show only weak reactivity whereas unpigmented cells in the outgrowth of the 2 to 3-day-old do so unequivocally strong reactivity (Fig. 1ab). The explants present in the latter culture also indicated an increased reactivity to this antibody. The similar assay with use of the MEBL-1 antibody disclosed that a large number of cells in the outgrowth of the 2 to 3-day-old culture show a detectable degree of the reactivity to it (Fig. 1cd). Careful observations of these reactive cells disclosed that most of them are installed with faintly melanized granular structures inside, suggesting that are melanogenesis-directed cells or melanoblasts. These neural crest-derived cells in culture gradually lost the reactivity to

HNK-1 and MEBL-1 antibodies with progress of melanogenesis, and fully melanized melanophores in the 3 to 4 day-old-culture were almost negligible in the reactivity to these antibodies (Fig. 2). Both of the faintly yellow-pigmented cells (presumably pterinogenesis-directed cells or xanthoblasts) and fully pigmented xanthophores appearing in the 2 to 3-day-old culture were poor in responding in the MEBL-1 antibody (Fig. 2cd). All these findings indicated that (1) goldfish neural crest derivatives are capable of reacting to the HNK-1 antibody at the beginning of their differentiation and (2) melanoblasts or its alike derived from goldfish neural crest express transiently during their differentiation even *in vitro* the epitopes which cross react with the MEBL-1 antibody.

When immunocytochemical assay using these two antibodies was carried out on cryosections of developing goldfish embryos, the reactivity of neural crest derivatives to them was essentially similar to that obtained on cultured cells *in vitro*, as described in the following section.

Distribution and fate of neural crest derivatives in developing goldfish embryos as revealed by labeling with the HNK-1 antibody

The neurulation in goldfish embryos started with formation of neural keel, a solid ridge of ectodermal cells at stage 18. The neural keel then formed neural tube by forming a cavity inside at stage 20, simultaneously yielding a cluster of neural crest (embryonic shield) cells outside along its dorsolateral ridge. Immunocytochemical assay on crossly cut cryosections of the trunk region of the embryos disclosed that a weak reactivity to the HNK-1 antibody become recognized first in cells distributing over the dorsal ridge of the neural keel at the stage 20 (Fig. 3a). With progress of development such reactive cells increased in numbers in the periphery of the neural tube facing to the somite (the dorsoventral route), whereas stayed in a limited number in the portions along the ectoderm (the lateral route), as shown with the stage 22 embryo (Fig. 3c). At this stage, faintly melanized melanophores become first recognized among the HNK-1 reactive cells, distributing rather densely over the trunk region. This well agreed with the previous observations on the stage of melanophore appearance by Kajishima (1960). At stage 24, the majority of positively reactive cells resided either in the ventral outer portion of the neural tube or in the midway of the dorsolateral routes in both side of the trunk (Fig. 3e). The former was presumed to be elements associated with peripheral nervous systems because of a firm settlement in the definite locations thereafter, continued reactivity to this antibody and an absence of melanized cells nearby. The latter was intermingled with melanized cells which showed a weak or almost negligible reactivity to this antibody. A tendency was observed with cells distributing along the ventral route that the reactivity to

this antibody is markedly decreased with melanogenesis (Fig. 3g). The distribution of HNK-1 positive cells in the embryos up to hatching was much or less similar to that at this stage, residing mostly in the ventral route and in a limited numbers along the ventral margin of the notochord. At these stages, fully melanized melanophores, which were negligible low in the reactivity to the antibody, were aligned in the dorsal edge of the somites forming a long stripe along the head to tail axis.

In both of the dorsolateral and ventral routes of HNK-1 reactive cells, melanophores made their first appearance in the position close to the neural tube at stage 22, but thereafter never resided in such position, being occupied exclusively with non-pigmented, HNK-1 reactive cells (Fig. 3c and 3eg). The longitudinal sections of the embryos at these stages disclosed that the HNK-1 positive cells and melanophores were aligned in a row running along the notochord to the endoderm, indicating the presence of the defined migratory route (Fig. 4). These findings indicated that (1) melanophores, in goldfish embryos migrated out from the neural crest (embryonic shield) into two routes for their distribution; (2) the vast majority of them took the ventral route, and (3) first moving melanophores develop the migratory routes as a pioneer, along which the successors travel to the settling sites without proceeding apparent melanogenesis.

Differentiation and migratory behavior of melanoblasts in goldfish embryos as revealed by labeling with the MEBL-1 antibody

Immunocytochemical staining disclosed that the reactivity to the MEBL-1 antibody became recognized first in the cells present near the dorsal tip of the neural tube of the embryos at stage 22 (Fig. 5a). Such reactivity appeared in those cells giving positive reaction to the HNK-1 with a slight delay to the expression of the reactivity to this antibody. Simultaneously with or slightly after the appearance of the reactivity to the MEBL-1, the onset of melanogenesis was observed (Fig. 5ab). This apparently indicated that the MEBL-1 antibody recognized the epitopes present in melanogenesis-directed cells or melanoblasts even in goldfish embryos. In embryos at stages thereafter, a large number of MEBL-1 positive cells were distributed in the ventral route, whereas a limited were so in the lateral (Fig. 5cd), both being intermingled with melanophores (Fig.6). It was noted that the MEBL-1 positive cells and melanophores were located in any routes under close typographical relation but never overlapped in the same one (Fig. 6), indicating a reciprocal relationship between the antigenicity to MEBL-1 and melanogenesis. Fig.7d indicates the coexistence of MEBL-1-positive cells and fully, melanized melanophores in the midventral corium of the embryo at stage 26. This would imply that melanoblasts have profound capability for migration to reach to the opposite side of the neural crest, and

that the differentiation of melanoblasts to melanophores brought about in any sites of the migratory routes, possibly responding to the cue from tissue environment.

Discussion

In this paper, we indicated by means of immunocytochemistry that the antibodies, HNK-1 (Erickson, et al., 1989) and MEBL-1 (Kitamura et al., 1992), can cross react respectively with neural crest cells and melanoblasts in goldfish embryos. And in this species, it is also disclosed that the epitopes reactive to the HNK-1 continue to exist in neural crest derivatives occurring at varying developmental stages right after neural crest formation to the expression of particular phenotype such as melanogenesis, whereas those by the MEBL-1 appear for a short period of time in differentiating melanoblasts slightly before or at the beginning of melanogenesis. Evidence obtained by combined application of these antibodies and microscopic observations on melanogenesis indicated that (1) a portion of neural crest cells differentiate into melanophores via melanoblasts, (2) they translocate to the destined addresses through two migratory routes, the dorsolateral and the ventral, and (3) all of undifferentiated neural crest-derivatives, melanoblasts and melanophores coexist in every sites of the migratory routes.

The findings reported in this paper had provided with a couple of significant knowledge on pigment cell differentiation: (1) The appearance of MEBL-1 positive cells among undifferentiated neural crest derivatives in the vicinity of the neural crest at earlier embryogenesis implies that the commitment of neural crest cell differentiation toward melanophores can take place immediately after their migration from the neural crest, as well as in later stages in various sites of the migratory routes; (2) The ubiquitous distribution of HNK-1 positive neural crest derivatives in the migratory routes, particularly in the lateral, over a wide range of developmental stages would indicate the presence of a resovoir of stem cell which provide with pigment cells to the corium when needed; (3) Frequent appearance of fully melanized melanophores in the migratory routes near the neural crest at earlier stages of development and their absence in subsequent stages in the similar sites which, instead, were occupied with numerous undifferentiated neural crest derivatives suggests the presence of a pioneer which opens a migratory route for the successors. The linear alignment of numerous neural crest derivatives along the migratory pathway would supplement this possibility; (4) The mixed distribution of undifferentiated neural crest derivatives, melanoblasts and melanophores even in a ventral site of the migratory routes implies that the differentiation of neural crest cells takes place in a site specific manner in which a local cue from the tissue provides with a trigger of the event.

So far, there are many reviews on neural crest migration (Graham, 2003; Tucker, 2004), but still the migration systems has not fully disclosed, Little information is available on the nature of such cues, through the possibility exists that some components of the extra cellular matrixes (Lofberg, 1980; Loring, 1982; Campbell, 1989) or the molecules provided by the tissue like melanization inhibiting factor (Fukuzawa & Ide, 1988; Fukuzawa & Bagnara, 1989) serve a cue of this kind.

It is extremely interesting to know from the phyletic standpoint that neural crest derivatives and melanoblasts are installed with the common epitopes recognized by the antibodies adopted in this study. It is well known that the HNK-1 antibody recognized the molecule belonging to immunoglobulin super-family, whereas two proteins of premelanosome like granules, 135K and 115K dalton, are recognized by the MEBL-1 (Kitamura et al., 1992).

In zebrafish, Kelsh et al., reported over 70 genes are involved in the process of pigment cell development and pattern formation using large numbers of mutants (1996). Some mutants showed poor melanoblast differentiation and migration (Kelsh et al., 2000; Quigley & Parichy; 2002). The Knockdown experiments of Foxd3 expression disclosed the gene expression is indispensable for several differentiated neural crest derivatives (jaw cartilage, peripheral neurons, glia and iridophores pigment cells) (Lister et al., 2006). Therefore, Foxd3 may not be required for determination of neural crest identity and migration of the derivatives. But such genetic analyses are certainly powerful tool to disclose the regulation mechanisms of neural crest migration and pigment cell differentiation.

In this study, much attention is focused on the differentiation of a melanoblast-melanophore system but not on that toward xanthophores or iridophores. Thus far, no evidence is obtained that the MEBL-1 antibody cross-react with these two non-melanogenic pigment cells or their blasts. This may indicate as a technical matter that the HNK-1 positive and MEBL-1 negative cells, which later become positive to the latter antibody or elicit pigmentation, are chromatoblasts. The development of unique probes characterizing these non-melanogenic cells will explore this line of studies.

Acknowledgments

Authors make deeply thanks to Tokyo Metropolitan Fisheries Experiment Station on Katsushika for kindly offer of goldfish eggs. A part of this research has been done by support from Keio University research Grand to T.A.

References

Bagnara, (1987) J.T. The neural crest as a source of stem cells.In: Developmental and evolutionary aspects of the neural crest. F.A.P. Maderson, ed. John Wiley & Sons. A Wiley-Interscience Publication, New York 57–87.

Bronner-Fraser, M. (1986) Analysis of the early stages of trunk neural crest migration in avian embryos using monoclonal antibody HNK-1. Dev. Biol., 115: 44–55.

Campbell, S. (1989) Melanogenesis of avian neural crest cells *in vitro* is influenced by external cues in the periorbital mesenchyme. Development, 106: 717–726.

Ciment, G. and Weston, J.A. (1982) Early appearance in neural crest and crest-derived cells of an antigenic determinant present in avian neurons. Dev. Biol., 93: 355–367.

Ciment, G. and Weston, J.A. (1985) Segregation of developmental abilities in neural-crest-derived cells: Identification of partially restricted intermediate cells types in the branchial arches of avian embryos. Dev. Biol., 111: 73–83.

Coles E.G, Gammill L.S, Miner J.H, Bronner-Fraser M. (2006) Abnormalities in neural crest cell migration in laminin alpha5 mutant mice. Dev Biol. Jan 1; 289 (1): 218–28.

Erickson, C.A., Loring, J.F. and Lester, S. (1989) Migratory pathways of HNK-1-immunoreactive neural crest cells in the rat embryo. Dev. Biol., 134: 112–118.

Fukuzawa, T. and Ide, H. (1988) A ventral localized inhibitor of melanization in *Xenopus laevis* skin. Dev. Biol., 129: 25–36.

Fukuzawa, T., and Bagnara, J.T. (1989) Control of melanoblast differentiation in amphibian by α-melanocyte stimulating hormone, a serum melanization factor, and a melanization inhibition factor. (1989) Pigment Cell Res., 2: 171–181.

Graham, A. (2003) The neural crest. Current Biology, 13 (10) R381–384.

Gammill, L.S., Gonzalez, C, Gu, C, and Bronner-Fraser, M. (2006) Guidance of trunk neural crest migration requires neuropilin 2/semaphorin 3F signaling. Development, Jan; 133(1): 99–106.

Halloran, M.C. & Berndt, J.D. (2003) Current progress in neural crest cell motility and migration and future prospects for the Zebrafish model system. Develop. Dynamics, 228: 497–513.

Kajishima, T. (1958) Regional differences in pigment cell formation of the embryonic shield of the goldfish. Embryologia, 4: 133–147.

Kajishima, T. (1960) The normal developmental stages of the goldfish, *Carassius auratus*. Jap. J. Ichthyology. VIII: 20–28.

Kelsh, R.N, Brand, M., Jiang, Y.J., Heisenberg, C.P., Lin, S., Haffter, P., Odenthal, J.,

Mullins, M.C., van Eeden, F.J., Furutani-Seiki, M., Granato, M., Hammerschmidt, M., Kane, D.A., Warga, R.M., Beuchle, D., Vogelsang. I., Nusslein-Volhard, C. (1996) Zebrafish pigmentation mutations and the processes of neural crest development. Development. 123: 369–89.

Kelsh, R.N., Schmid. B., and Eisen, J.S., Genetic analysis of melanophore development in zebrafish embryos. Dev Biol. 2000 15; 225 (2): 277–93.

Kitamura, K., Taniguchi-Hayashi, K., Sezaki, M., Yamamoto, Y. and Takechi, T. (1992) Arian neural crest cells express a melanogenic trait during early migration from the neural tube: observations with the new monoclonal antibody, "MEBL-1". Development 114: 367–378.

Le Douarin, N. M. (1980) Migration and differentiation of neural crest cells. In: Current Topics in developmental Biology, 16; 32–85.

Le Douarin, N. M. (1982) The neural crest. Cambridge University Press, Cambridge and London.

Lister, J.A., Cooper, C., Nguyen, K., Modrell, M., Grant, K., and Raible, D.W. (2006) Zebrafish Foxd3 is required for development of a subset of neural crest derivatives.1; 290 (1): 92–104.

Lofberg, J. Ahofors, K. and Fallstrom, C. (1980) Neural crest cell migration in relation to extracellular matrix organization in the embryonic axolotl trunk. 75: 148–167.

Loring, J., Glimelius, B. and Weston, J. A. (1982) Extracellular matrix materials influence quail neural crest cell differentiation *in vitro*. Develop. Biol., 90: 165–174.

Matsumoto, J., Akiyama, T. and Kitamura, K. (1989) Immunocytochemical characterization of neural crest-associated markers appearing in goldfish erythrophoroma cells under differentiation *in vitro*. Zool. Sci., 6: 1143.

Newgreen, D. F., Powell, M. E. and Moser, B. (1990) Spatiotemporal changes in HNK-1/L2 glycocongugates in avian embryo somite neural crest cells. Develop. Biol., 139: 100–120.

Obika, M. (1963) Association of pteridines with amphibian larval pigmentation and their biosynthesis in developing chromatophores. Dev. Biol., 6: 99–112.

Quigley, I.K. and Parichy, D.M. (2002) Pigment Pattern formation in zebrafish: A model for developmental genetics and the evolution of form. Microscopy res. and Tech., 58: 442–455.

Richards, C.M. and Bagnara, J.T. (1967) Expression of specific pteridines in neural crest transplants between *Pleurodeles* and axolotl. Dev. Biol., 15: 334–347.

Sadaghiani, B. and Vielkind, J.R. (1989) Neural crest development in Xiphophorus fish: Scanning electron and light microscopic studies. Development, 105: 487–504.

Sadaghiani, B. and Vielkind, J. R. (1990a) Explanted fish neural tubes give rise to

differentiating neural crest cells. Develop. Growth and Differ, 32: 513–520.

Sadaghiani, B. and Vielkind, J.R. (1990b) Distribution and migration pathways of HNK-1 immunoreactive neural crest cells in teleosts fish embryos. Development, 110: 197–207.

Serbedzija, G.N., Bronner-Fraser, M. and Fraser, S. E. (1989) A Vital dye analysis of the timing and pathways of avian trunk neural crest cell migration development, 106: 809–816.

Serbedzija, G.N., Fraset, S. E. and Bronner-Fraser, M. (1990) Pathways of trunk neural crest cell migration in the mouse embryo as revealed vital dye labeling. Development, 108: 605–612.

Tucker, R.P. (2004) Neural crest cells: a model for invasive behavior. Intl. J. Biochem & Cell Biol. 36: 173–177.

Tucker, G.C., Aoyama, H., Lipinski, M., Turz, T., and Thiery, J. P. (1984) Identical reactivity of monoclonal antibodies HNK-1 and NC-1: Conservation in vertebrates on cells derived from neural prinordium and on some leukocytes. Cell Differ. 14: 223–230.

Weston, J. A. (1963) A radioautographic analysis of the migration and localization of trunk neural crest cells in the chick. Develop. Biol., 6: 279–310.

Weston, J. A. (1970) The migration and differentiation of neural crest cells. Adv. Morphogenesis, 8: 41–114.

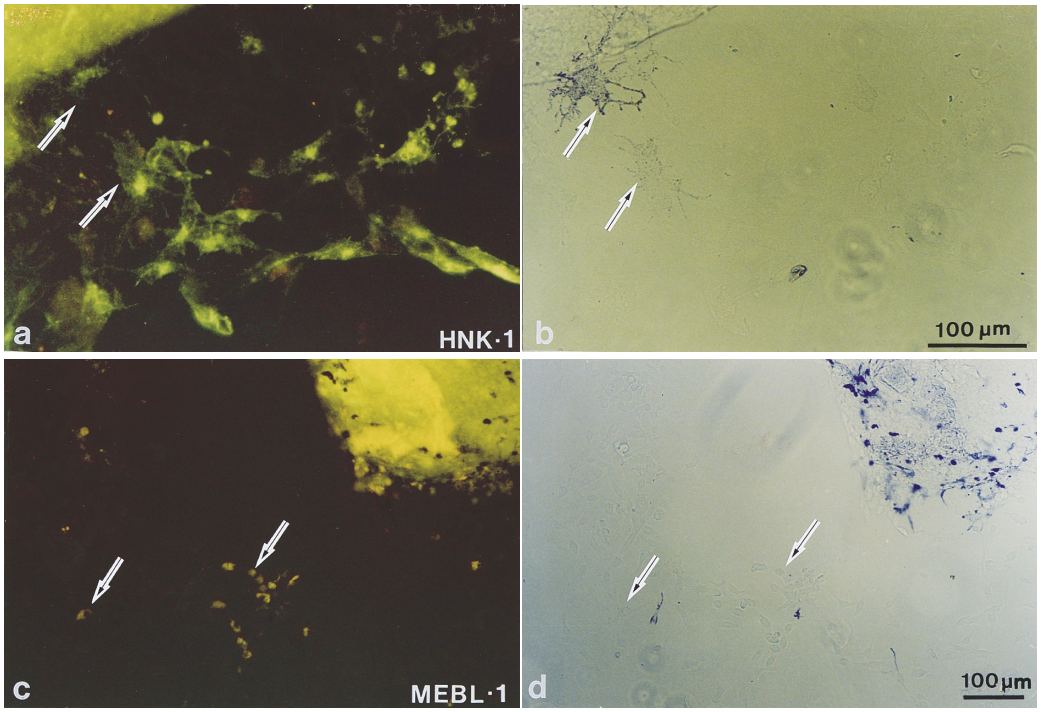

Fig.1 Immunofluorescence patterns of emerging cells from the neural crest (embryonic shield) explants obtained from goldfish neurula (stage 15) with labeling by the HNK-1 or MEBL-1 antibody. Under labeling with HNK-1, almost all unpigmented cells migrated out from an explant are stained densely whereas melanophores (arrows) at the onset of melanogenesis weakly (a, b). And under MEBL-1, only faintly melanized cells are decisively stained (c, d; arrows). Both are assayed on the 2-day-old culture after explanation. (a, c) ; immunofluorescence, (b, d) ; phase contrast. Scale bar: 100 μm.

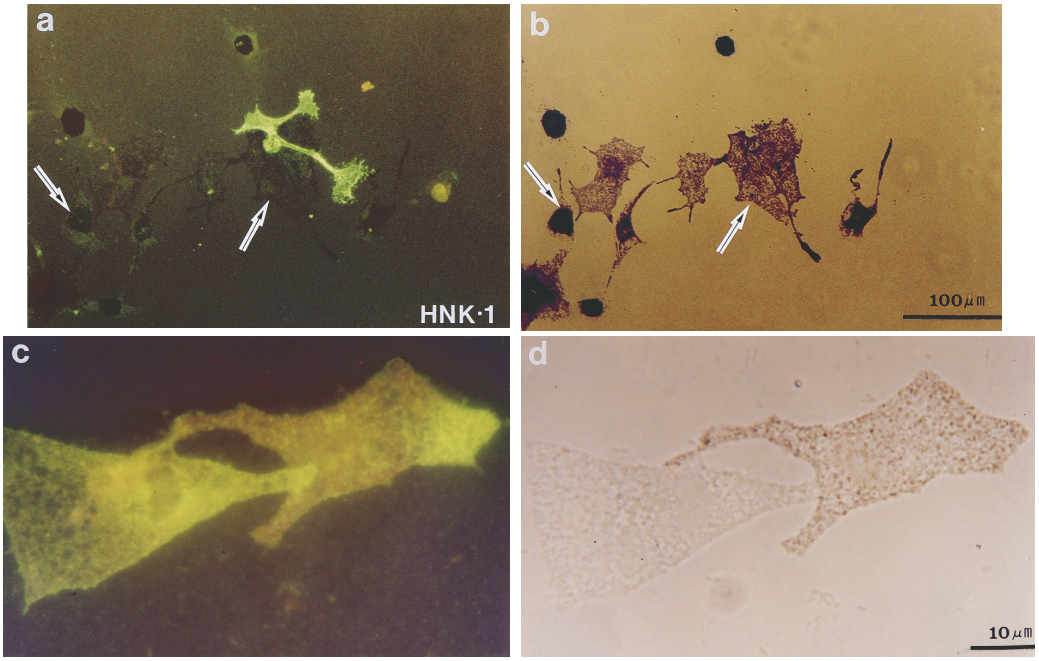

Fig.2 Immunofluorescence patterns of emerging cells derived from organ culture as same as Fig. 1. labeled with the HNK-1 or MEBL-1 antibody. Fully melanized cells lost the reactivity with HNK-1 from 3-4 day old culture (a, b). Presumptive erythrophore (right in c, d) and xanthophore (left in c, d) show similar poor responses with the MEBL-1 antibody. a, c; immunofluorescence, b, d; phase contrast. Scale bar; b: 100 μm, d: 10 μm

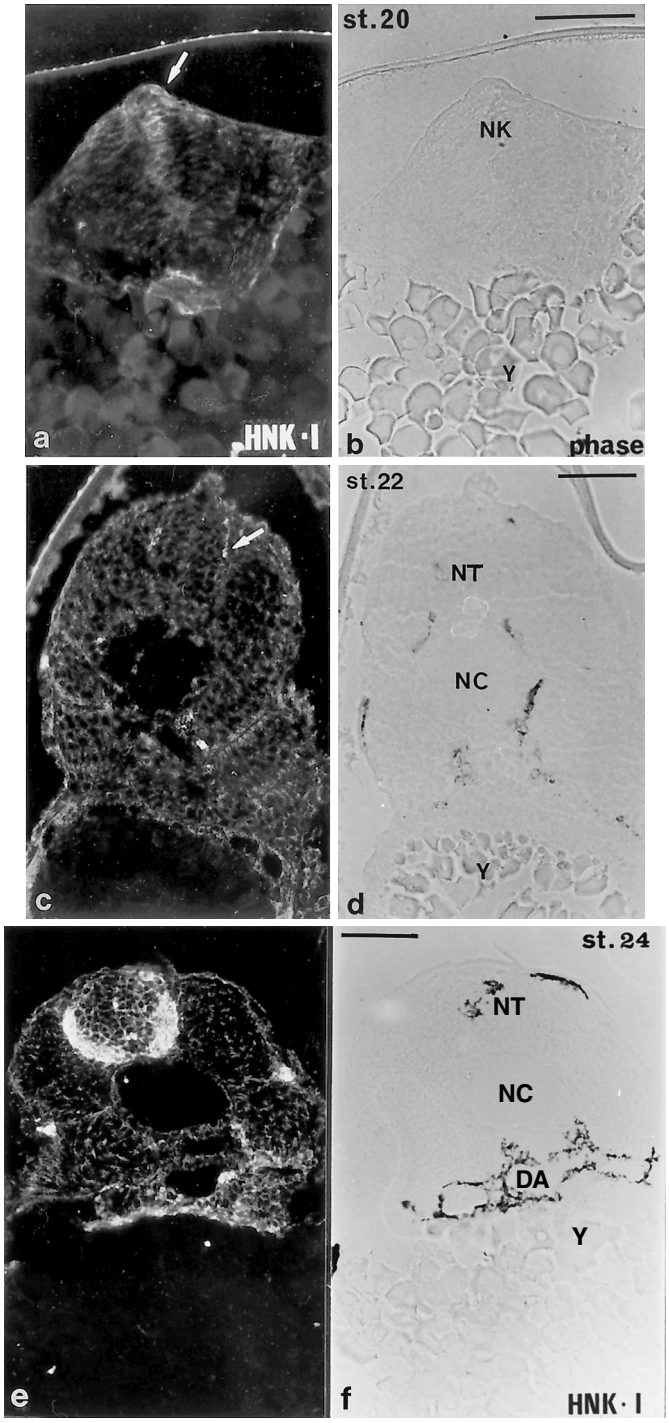

Fig.3 (a-f)

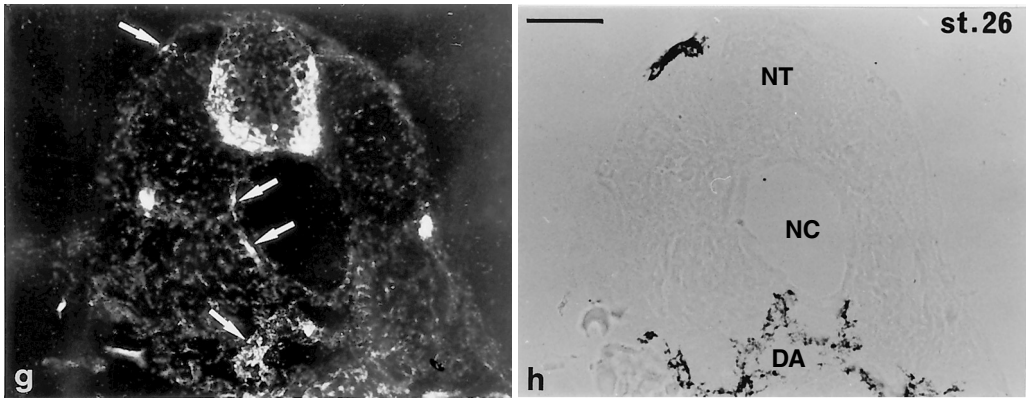

Fig.3 (g-h)

Fig.3 Immunofluorescence patterns on cross sections of goldfish embryo (trunk region) from labeling with HNK-1 (a, c, e, and g) and their images under a phase contrast (b, d, f, h). (a) Stage 20. Note the presence of HNK-1 labeling in the dorsal ridge (arrow) and the periphery of the presumptive neural tube (neural keel; NK). (c) Stage 22. Note the presence of clear HNK-1-positive cells. Faintly melanized cells locate in the lateral boundaries of the neural tube and the periphery of the notochord and the somites (d). (e) Stage 24. Clear positive cells locate in the both sides of the ventral outer area in neural tube, the lateral corners of the somites in the both sides of the trunk and an area surrounded by the somites and yolk sac. See the text for further explanation. (g) Stage 26. Note the presence of distinct labeling in the both lateral sides of the neural tube and spots locates at lateral sides of somites, possibly nerve ganglions. The labeling is also recognized along the notochord, the corium and dorsal aorta indicating migratory neural crest derivatives in the lateral and dorsal routes (arrows). A large number of melanophores (h) reside in the space between the somites and yolk sac and surrounding dorsal aorta, being intermingled with HNK-1 positive cells. NK: neural keel, NT: neural tube, NC: notochord, DA: dorsal aorta, Y: yolk, S: somite, Scale bar: 100 μm.

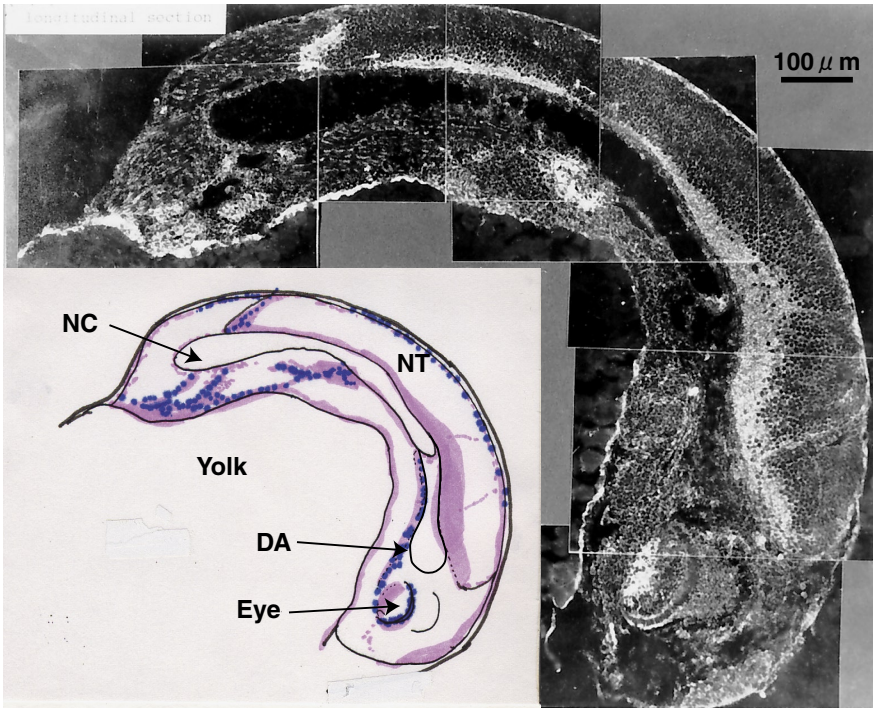

Fig.4 Immunofluorescence of a longitudinal section of the goldfish embryo (stage 26) labeling with HNK-1 antibody (top). Note that labeled cells are located in a close relation with melanophores as shown in illustration superimposed with the locations of melanophores (blue spot) on the HNK labeling (violet zone and spots) of the same specimen (bottom). Distinctly labeled cells along the bottom of neural tube are considered to be mostly elements associated with nervous system. Well matched locations of melanophores with HNK-1 positive zone show a close relation of HNK-1 positive cells and melanophores. NT: neural tube, NC: notochord, DA: dorsal aorta, Scale bar: 100 μm.

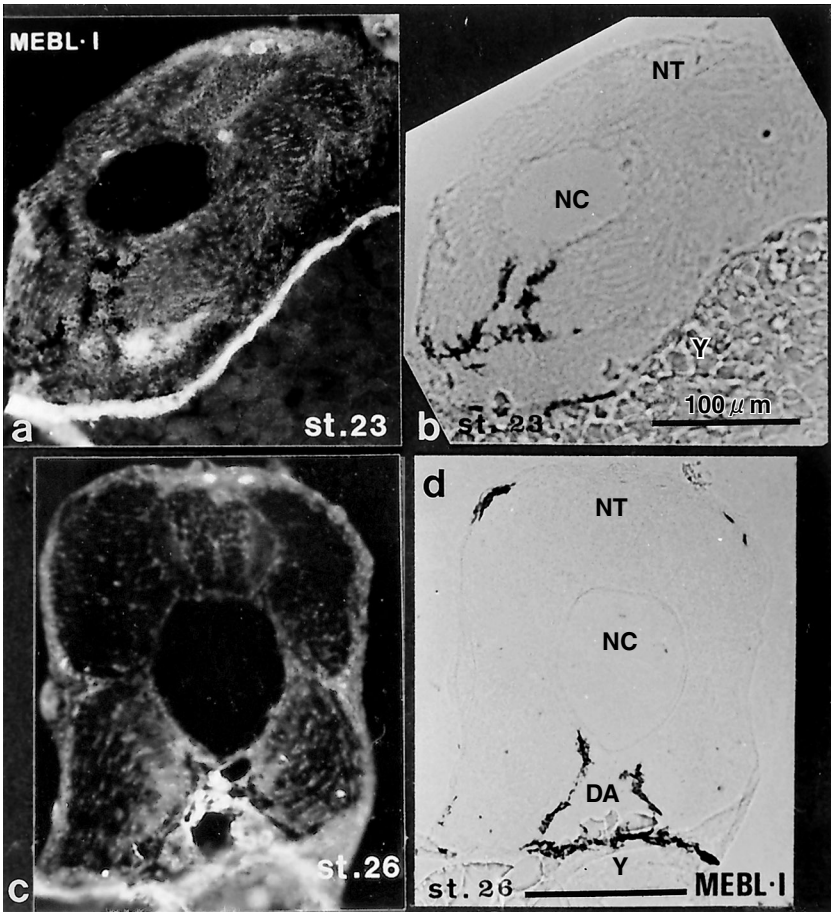

Fig.5. Immunofluorescence of cross sections of goldfish embryo labeled with the MEBL-1 antibody (a, c) and their phase contrast image (b, d). (a) Stage 23. Note the presence of few cells with distinct labeling along the dorsal tip of the presumptive neural tube and the notochord. (c) Stage 26. Note a cell with clear labeling at the entrance of the dorsolateral route. NT: neural tube, NC: notochord, DA: dorsal aorta, Y: yolk, S: somite, Scale bar: 100 μm.

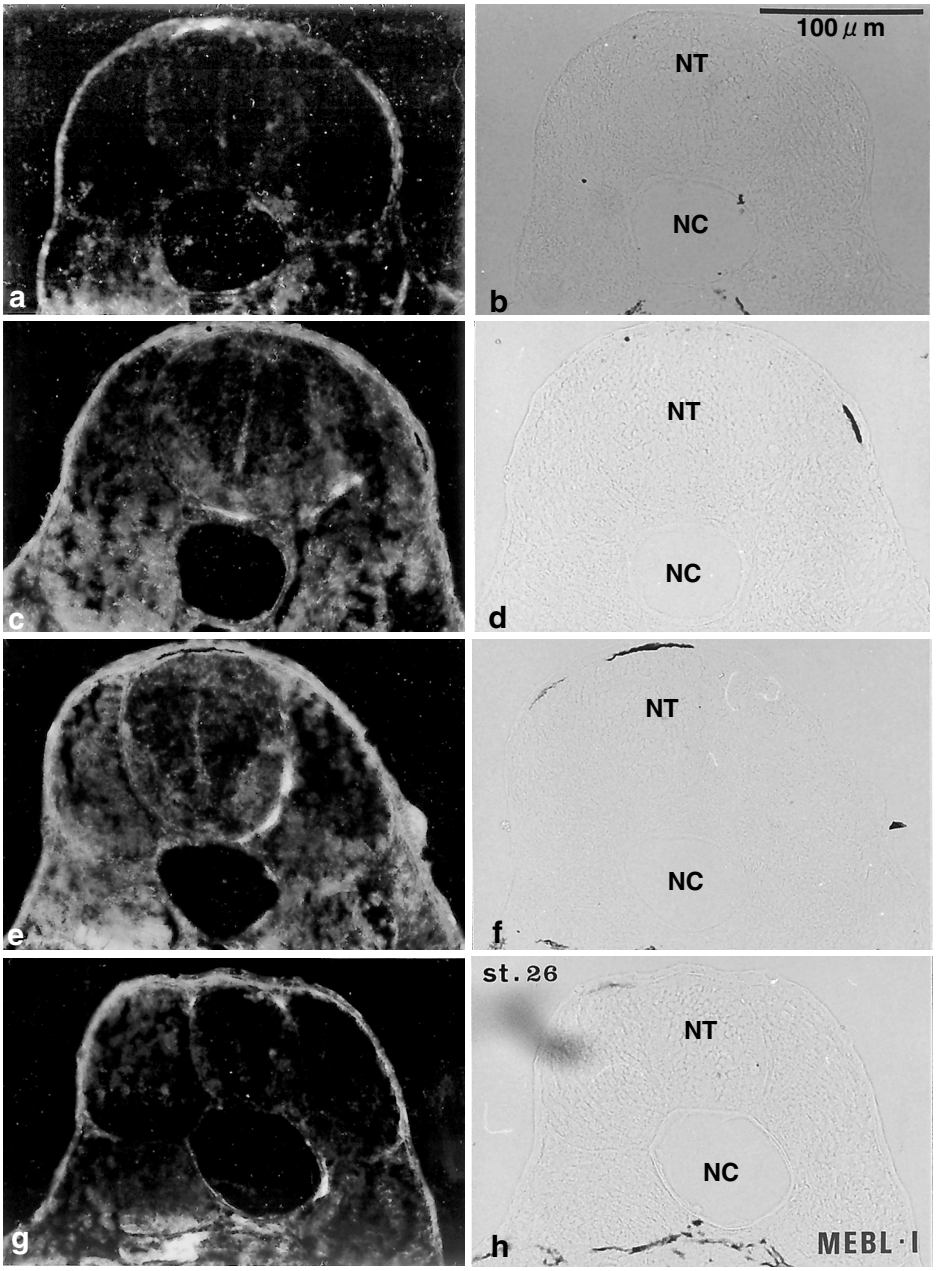

Fig.6 Stage 26. Note the distributions of several cells with clear labeling in the dorsoventral route and a limited number of cells in the dorsolateral route. These MEBL-1 positive pattern shows that melanophores in goldfish migrate through both of dorsoventral and dorsolateral routes. NT; neural tube, NC; notochord, S; somite, Y; yolk. Scale bar: 100 μm.

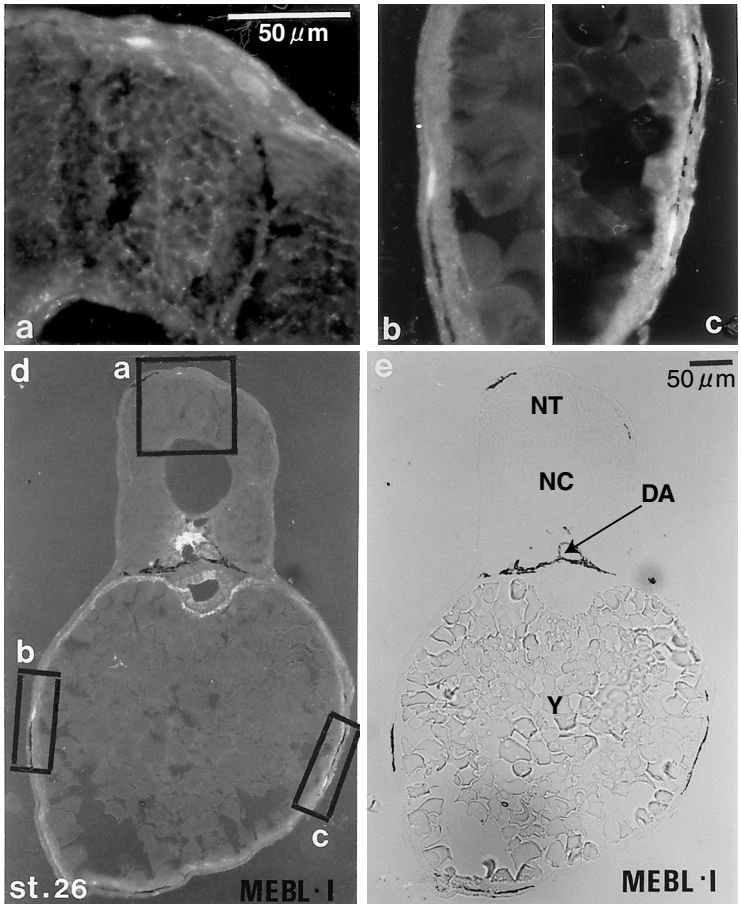

Fig.7 Stage 26. Location of the labeled cells in embryo with MEBL-1 and the enlarged views. (a, b, c) The enlarged images of the same area in the rectangles, a, b, and c in d. Note a close topographical association between labeled cells are melanophores. NT: neural tube, NC: notochord, DA: dorsal aorta, Y: yolk, S: somite, Scale bar: 50 μm.