

Ascidian Neural Crest-Like Cells: Phylogenetic Distribution, Relationship to Larval Complexity, and Pigment Cell Fate

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ABSTRACT Migratory neural crest-like cells, which express the cell surface antigen HNK-1 and develop into pigment cells, have recently been identified in the ascidian *Ecteinascidia turbinata*. Here we use HNK-1 expression as a marker to determine whether neural crest-like cells are responsible for pigment development in diverse ascidian species. We surveyed HNK-1 expression and tyrosinase activity in 12 ascidian species, including those with different adult organizations, developmental modes, and larval sizes and complexities. We observed HNK-1 positive cells in every species, although the timing of HNK-1 expression varied according to the extent of larval complexity. HNK-1 expression was initiated during the late tailbud stage in species in which adult features are formed precociously in large complex larvae. In contrast, HNK-1 positive cells did not appear until the swimming tadpole or juvenile stage in species with small simple larvae in which most adult features appear after metamorphosis. Double labeling experiments indicated that HNK-1 and tyrosinase are expressed in the same subset of pigment-forming mesenchymal cells in species with complex or simple larvae. In addition, the absence of HNK-1 and tyrosinase expression in albino morphs of the colonial ascidian *Botryllus schlosseri* suggested that the major fate of neural crest-like cells is to become pigment cells. The results suggest that ascidian neural crest-like cells and vertebrate neural crest cells had a common origin during chordate evolution and that their primitive function was to generate body pigmentation. *J. Exp. Zool. (Mol. Dev. Evol.)* 306B:470–480, 2006.

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The morphological complexity of vertebrates is due largely to the many contributions of the neural crest. Neural crest cells originate from the border of the neural and non-neural ectoderm, migrate extensively through the embryo, and generate a wide range of derivatives, including the peripheral nervous system, craniofacial bone and cartilage, and glial, endocrine, and pigment cells (Hall, '99; Le Douarin and Kalcheim, '99). The fundamental role of neural crest derivatives in vertebrate evolution inspired studies to identify homologous cells in other chordates. In amphioxus and several ascidian species, cells at the neural plate border were shown to express some of the regulatory genes characteristic of vertebrate neural crest but these cells did not undergo extensive migration during the embryonic period (Ma et al., '96; Corbo et al., '97; Wada et al., '97; Sharman et al., '99; Holland and Holland, 2001; Wada, 2001). Consequently, it was concluded that migratory neural crest cells evolved in the vertebrates (Baker and Bronner-Fraser, '97; Meulemans and Bronner-Fraser, 2005), although

a “latent” neural crest may be present in other chordates (Stone and Hall, 2004).

Ascidians have a life cycle consisting of a dispersal phase, in which larvae are produced during embryonic development, and a sessile phase, which begins after metamorphosis (Jeffery and Swalla '97). The early searches for ascidian neural crest homologues were restricted to *Molgula oculata*, *Ciona intestinalis*, and *Halocynthia roretzi* (Ma et al., '96; Corbo et al., '97; Wada et al., '97), which exhibit rapid development and relatively small larvae with minimal predifferentiation of adult structures. The small size of these embryos discouraged approaches such as cell tracing and transplantation, which have been instrumental in defining migratory neural crest

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cells in vertebrates (Hall, '99; Le Douarin and Kalcheim, '99). In contrast, many other ascidian species have large structurally complex larvae (Berrill, '35, '47, '50) produced by adulation: the precocious differentiation of adult tissues and organs in the larval trunk (head) during embryonic development (Jeffery and Swalla, '92). In the ascidian *Ecteinascidia turbinata*, which exhibits extreme adulation and "giant" larvae, migratory cells originate from the dorsal neural tube region, express the vertebrate neural crest markers *zic2* (Elms et al., 2003) and HNK-1 (Tucker et al., '84; Vincent and Thierry, '84), and differentiate into body pigment cells (Jeffery et al., 2004). These studies suggested that migratory neural crest-like cells could be present outside of the vertebrates.

There are two ways to explain the presence of neural crest-like cells in *Ecteinascidia* embryos. First, migratory cells that express HNK-1 and develop into pigment cells may have evolved independently in ascidians. In this case, ascidian HNK-1 positive cells would have evolved neural crest-like features through convergence. Second, pigment-forming HNK-1 positive cells could be a general characteristic of ascidians. This possibility would be consistent with a common origin of ascidian neural crest-like cells and vertebrate neural crest cells during chordate evolution.

To distinguish between these possibilities, we have used the cell surface glycoprotein HNK-1, which is expressed by migratory neural crest cells in many groups of vertebrates (Tucker et al., '84; Vincent and Thierry, '84; Bronner-Fraser, '86; Erickson et al., '89; Hirata et al., '97), as a marker to survey neural crest-like cells in diverse ascidian species, including those with colonial and solitary adult organization, different modes of development, and large and small larvae. As a way to test the pigment-forming capacity of ascidian neural crest-like cells, we have developed procedures to detect HNK-1 and tyrosinase, the key enzyme in melanogenesis, in the same cells. Finally, to assess whether ascidian neural crest-like cells have developmental fates in addition to pigment formation, we have compared HNK-1 and tyrosinase expression in colored and albino morphs of the colonial ascidian *Botryllus schlosseri*.

MATERIALS AND METHODS

Animals

Styela clava, *B. schlosseri*, *Botrylloides diegenisis*, *Perophora viridis*, *Molgula citrina*, *Didemnum candidum*, *Amaroucium (Aplidium) constellatum*,

and *C. intestinalis* were collected at Woods Hole, MA and/or provided by the Aquatic Resources Division, Marine Biological Laboratory, Woods Hole, MA. *Botryllus* black and albino colony morphs were collected in Eel Pond, Woods Hole, MA or Sandwich Harbor, Sandwich, MA. *Morchelium argus*, *Molgula socialis*, *C. intestinalis*, and *Molgula occulta* were collected at Roscoff, France and/or provided by collectors at Station Biologique, Roscoff, France. Gulf Specimens Inc. (Panacea, FL) provided *E. turbinata*. Embryos were obtained from ovoviparous species (*Ciona*, *Styela*, *M. socialis*, and *M. occulta*) by in vitro fertilization. Tailbud embryos and mature larvae were obtained from viviparous species (all others) by dissection. Embryos and larvae were cultured by conventional methods (Grave, '59; Costello and Henley, '71; Jeffery et al., 2004).

HNK-1 expression

One of two methods was used for immunostaining depending on whether HNK-1 monoclonal antibody (anti-human HNK-1/CD57, BD Biosciences Pharingen, San Jose, CA) and its detection reagents were able to penetrate the larval and/or adult tunic.

Penetration was possible in *Botryllus*, *Botrylloides*, *Perophora*, *Ecteinascidia*, and *Ciona*. In these species, whole specimens were fixed in 4% paraformaldehyde (PF; pH 7.2 in PBS) overnight, dehydrated through an ethanol series to 100%, and stored in 100% methanol. They were stained with HNK-1 antibody (1:10 dilution) and antibody-antigen complexes were detected with a biotinylated goat anti-mouse IgM secondary antibody (1:100; Vector Laboratories, Burlingame, CA) using the Vectastain ABC Peroxidase kit (Vector Laboratories) as described by Jeffery et al. (2004).

Penetration of the larval and/or adult tunic was inefficient or not possible in *Amaroucium*, *Didemnum*, *Morchelium*, *M. socialis*, *M. citrina*, *M. occulta*, and *Styela*. In these species, specimens were fixed in 4% PF as above, dehydrated to and stored in 100% ethanol, embedded in polyester wax (Steedman, '57; Norenburg and Barrett, '87), and sectioned at 10 μ m. The sections were stained for 1 hr at 18°C with HNK-1 antibody (1:10 to 1:20 in PBS, 18°C), washed several times in PBS, incubated for 1 hr at 18°C with rhodamine-conjugated goat anti-mouse IgM secondary antibody (1:100 in PBS, 18°C; Chemicon

International, Temecula, CA), washed in PBS, and mounted for viewing.

Controls were treated with non-immune mouse serum (1:10 dilution in PBS; AbCam, Cambridge, MA), calretinin antibody (1:50 in PBS; Sigma, St. Louis, MO), or acetylated alpha-tubulin antibody (1:20 in PBS; Sigma) and staining was assessed with an appropriate second antibody. The stained specimens were viewed with conventional light or fluorescence microscopy in PBS or 80% glycerol and photographed.

Tyrosinase expression

Specimens were fixed in 4% formalin-sea water for 30 min at 4°C, washed several times in sea water to remove the fixative, and subjected to the DOPA oxidase assay as described previously (Swalla and Jeffery, '91; Jeffery, 2004). Controls were assayed in the presence of the tyrosinase inhibitor 1-phenyl-2-thiourea (1 mM; Sigma). The assayed specimens were washed in PBS, viewed by light microscopy, and photographed.

HNK-1 and tyrosinase double labeling

To detect HNK-1 expression and tyrosinase activity in the same specimens, they were fixed and subjected to the DOPA oxidase assay as described above, washed several times in PBS, post-fixed in 4% PF overnight, dehydrated to 100% ethanol, embedded in polyester wax, and sectioned at 10 µm. The sections were stained with HNK-1

antibody and antibody-antigen complexes were detected using rhodamine-conjugated anti-mouse IgM as described above. After completion of antibody staining, sections were viewed by light microscopy to reveal tyrosinase-positive cells and by fluorescence microscopy to detect HNK-1 positive cells.

RESULTS

HNK-1 expression

HNK-1 expression was surveyed in 12 different ascidian species: three aplousobranchs, three phlebobranchs, and six stolidobranchs. The ascidian panel includes solitary and colonial species, species with different developmental modes, species with large and small embryos, and species with different larval complexities. HNK-1 stained cells were observed in each species (Table 1). Controls probed with non-immune serum or other antibodies did not show reactivity or reactivity did not match that detected by HNK-1 antibody.

Figure 1 shows examples of HNK-1 expression in some of the aplousobranch (Fig. 1A and A'), phlebobranch (Fig. 1B and C), and stolidobranch (Fig. 1D, E, F and F') species. In *A. constellatum*, an aplousobranch with adulation and large, structurally complex larvae (Grave, '21), HNK-1 expression was detected in mesenchyme cells scattered throughout the trunk of late tailbud embryos (Fig. 1A and A'). In *Morchelium argus*,

TABLE 1. Survey of HNK-1 and tyrosinase expression in ascidians

Suborder <i>Species</i>	Organization	Egg size (µm)	Developmental mode	HNK-1 cells			BTPC		
				Embryo ¹	Larva	Juvenile	Embryo	Larva	Juvenile
Aplousobranchia									
<i>Amaroucium constellatum</i>	Colonial	250	Adultation	+ ²	+	nd	+	+	nd
<i>Didemnum candidum</i>	Colonial	280	Adultation	+	+	nd	+	+	nd
<i>Morchelium argus</i>	Colonial	340	Adultation	+	+	nd	+	+	nd
Phlebobranchia									
<i>Ciona intestinalis</i>	Solitary	160	Simple	-	+	+	-	-	+
<i>Ecteinascidia turbinata</i>	Colonial	720	Adultation	+	+	nd	+	+	nd
<i>Perophora viridus</i>	Colonial	240	Adultation	+	+	nd	+	+	nd
Stolidobranchia									
<i>Botryllus schlosseri</i>	Colonial	280	Adultation	+	+	-	+	+	-
<i>Botryloides diegensis</i>	Colonial	250	Adultation	+	-	-	+	-	nd
<i>Molgula citrina</i>	Solitary	210	Adultation	+	+	nd	+	+	nd
<i>Molgula socialis</i>	Solitary	150	Simple	-	-	+	-	-	+
<i>Molgula occulta</i>	Solitary	110	Simple, anural	-	-	+	-	-	+
<i>Styela clava</i>	Solitary	150	Simple	-	-	+	-	-	+

¹Tailbud stage.

²+: positive result; -: negative result; Nd: no data.

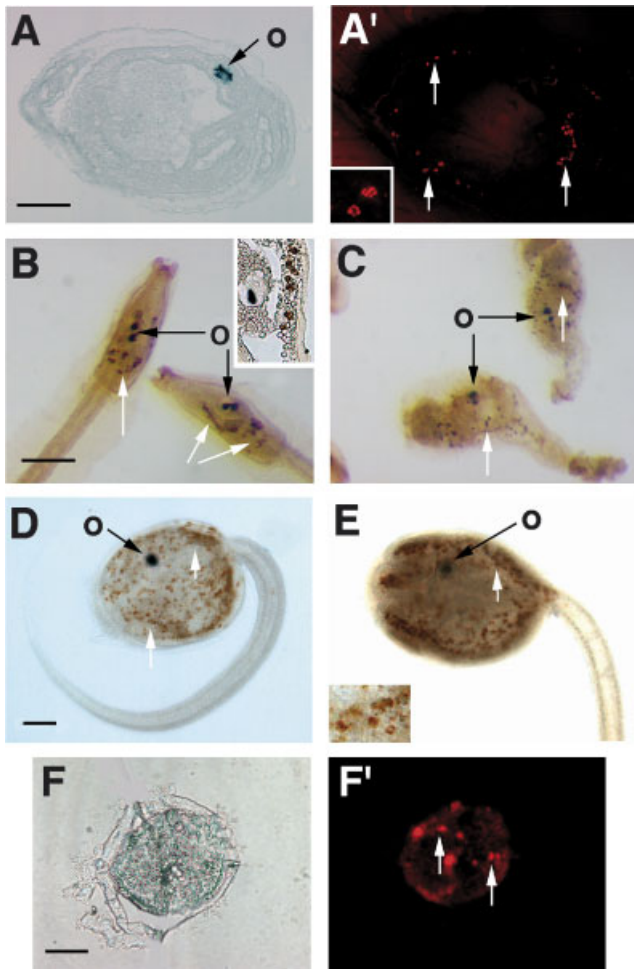


Fig. 1. HNK-1 expression: (A, A', F, F') detection of HNK-1 stained cells in sections using a fluorescent secondary antibody; (B–E) detection of HNK-1 stained cells in whole mounts using a biotinylated secondary antibody; (A–A') HNK-1 stained cells in an *Amaroucium* tailbud embryo; (A) bright field; (A') fluorescence. Inset in (A'): 4 \times ; (B, C) HNK-1 stained cells in *Ciona* tadpoles (B; dorsal views) and 7-day juveniles (C; bottom lateral and top dorsal views). Inset in (B): HNK-1 stained cells viewed in section at 4 \times ; (D, E) HNK-1 stained cells in a *Botryllus* tailbud stage embryo (D, lateral view) and tadpole larva (E, ventral view). Inset in (E): 4 \times ; (F, F') HNK-1 stained cells in a *M. occulta* 3-day juvenile; (F) bright field; (F') fluorescence. White arrows: HNK-1 positive cells. o (black arrows): Melanized otolith and ocellus brain sensory organs. Scale bars in (A) and (D) are 100 μ m (same magnification in D, E) and in (B) and (C) are 50 μ m (same magnification in B, C).

another aplousobranch with large complex larvae (Berrill, '50), HNK-1 reactivity was observed in both larval trunk mesenchyme and tunic cells (see Fig. 3A'). In *B. schlosseri*, a stolidobranch with large complex larvae (Grave and Woodbridge, '24), HNK-1 was expressed in trunk mesenchyme cells during the tailbud and swimming larval stages (Fig. 1D and E; see also Figs. 3D' and 4E, G).

In *Botrylloides diegensis*, another stolidobranch with adulation (Berrill, '47), HNK-1 expression was also present in late tailbud embryos but disappeared as pigment cells began to differentiate in swimming larvae (Table 1). In contrast, HNK-1 stained cells were first detected after larval hatching in the phlebobranch *C. intestinalis*, which exhibits small simple tadpoles lacking appreciable adult features. In *Ciona* larvae, HNK-1 expression was observed in a relatively small number of trunk mesenchyme cells located posterior and lateral to the brain sensory vesicle (Fig. 1B). After metamorphosis, HNK-1 stained cells were dispersed throughout the body and tunic of *Ciona* juveniles (Fig. 1C; also see Fig. 3B').

In each of the species described above, HNK-1 expression was observed in a subset of trunk mesenchyme cells, and sometimes in tunic cells, but was absent in the tail, as originally demonstrated in *Ecteinascidia* (Jeffery et al., 2004). Moreover, these cells were more numerous in species with complex larvae (e.g., the three aplousobranchs, *Botryllus*) than in those with simple larvae (e.g. *Ciona*, stolidobranchs other than *Botryllus* and *Botrylloides*, see below). The latter is illustrated by comparing *Botryllus* tadpoles, which have abundant HNK-1 staining cells (Fig. 1E), to those of *Ciona*, which have much fewer of these cells (Fig. 1B).

In stolidobranchs with small simple larvae, such as *S. clava* and *M. socialis*, HNK-1 positive cells could not be detected in tailbud stage embryos or larvae but were observed in juveniles after metamorphosis (Table 1). Likewise, in *M. occulta*, a stolidobranch with small anural (tailless) larvae of low structural complexity (Jeffery and Swalla, '92), HNK-1 stained cells were absent from larvae but detected throughout the body in post-metamorphic juveniles (Fig. 1F and F'). Finally, *M. citrina*, a stolidobranch with adulation (Grave, '26; Swalla et al., '94), showed HNK-1 expression in mesenchyme cells during the late tailbud stage (see Fig. 3C').

Tyrosinase expression

Pigment cell precursors were examined in 12 different species using the DOPA oxidase assay. The DOPA oxidase assay detects the activity of tyrosinase, the key enzyme in melanogenesis. Tyrosinase-positive cells were observed beginning at different times between the tailbud and juvenile stages in various species (Table 1). These cells are referred to here as body tyrosinase-positive cells

(BTPC) to distinguish them from the melanin-containing otolith and ocellus brain sensory cells, which express tyrosinase at earlier developmental stages (Jeffery, 2004). Controls in which the DOPA oxidase assay was conducted in the presence of the tyrosinase inhibitor 1-phenyl-2-thiourea did not show melanin deposition in BTPC, indicating that the assay specifically detected tyrosinase.

In three alousobranchs, the phlebobranchs *Ecteinascidia* and *P. viridis*, and the stolidobranchs *Botryllus*, *Botrylloides*, and *M. citrina*, which show adulation and complex larvae, BTPC were present in late tailbud embryos and swim-

ming larvae (Table 1). For example, in *Morchelium* (Fig. 2A) and *Botryllus* (Fig. 2D), BTPC were detected in the larval trunk mesenchyme and/or tunic but not in the tail. Some of these species have highly pigmented larvae (e.g. *Ecteinascidia*, *Botrylloides*) or juveniles (*Botryllus*). In these species, BTPC disappeared as body pigment cells differentiated (see Fig. 3F–G'), supporting the role of BTPC as pigment cell precursors.

In stolidobranchs with simple larvae, BTPC were absent in tailbud stage embryos and larvae but could be detected after metamorphosis (Table 1). For example, *Ciona* and *M. occulta* larvae were devoid of BTPC (Fig. 2B and E) but they appeared in post-metamorphic juveniles (Fig. 2C and F). In addition, BTPC were generally more abundant in species with complex larvae (e.g., *Botryllus*; Fig. 2D) than those with simple larvae (*Ciona*, Fig. 2C, *M. occulta*, Fig. 2F). Thus, BTPC are present in diverse ascidian species and resemble HNK-1 positive cells in their localization, abundance, and timing of tyrosinase expression.

HNK-1 positive cells, BTPC, and pigmentation

Two approaches were used to determine the relationship between HNK-1 and tyrosinase expression. First, double labeling experiments were conducted to determine whether the same or different cells express tyrosinase and HNK-1 in various ascidian species. Second, *Botryllus* natural pigmentation morphs were used to investigate the relationship between HNK-1 expression, tyrosinase expression, and body pigmentation.

To detect HNK-1 and tyrosinase expressing cells, tadpoles or juveniles of four different species were subjected to the DOPA oxidase assay, sectioned, and the sections were stained with HNK-1 antibody. Substantial numbers of double-labeled cells were observed in *Morchelium* (Fig. 3A and A'), *M. citrina* (Fig. 3C and C'), and *Botryllus* (Fig. 3D and D') larvae and in post-metamorphic *Ciona* juveniles (Fig. 3B and B'), indicating that BTPC express HNK-1. In addition, a few tyrosinase-negative, HNK-1 stained cells were detected at early developmental stages in some species. For example, in *Ciona* HNK-1 stained cells were tyrosinase negative in tadpoles (compare Fig. 1B and 2B) but all BTPC expressed HNK-1 in 7-day juveniles (Fig. 3B and B'). Furthermore, in some of these species, HNK-1 expression was not seen in some BTPC (Fig. 3D', F', and F', black arrows without corresponding white arrows).

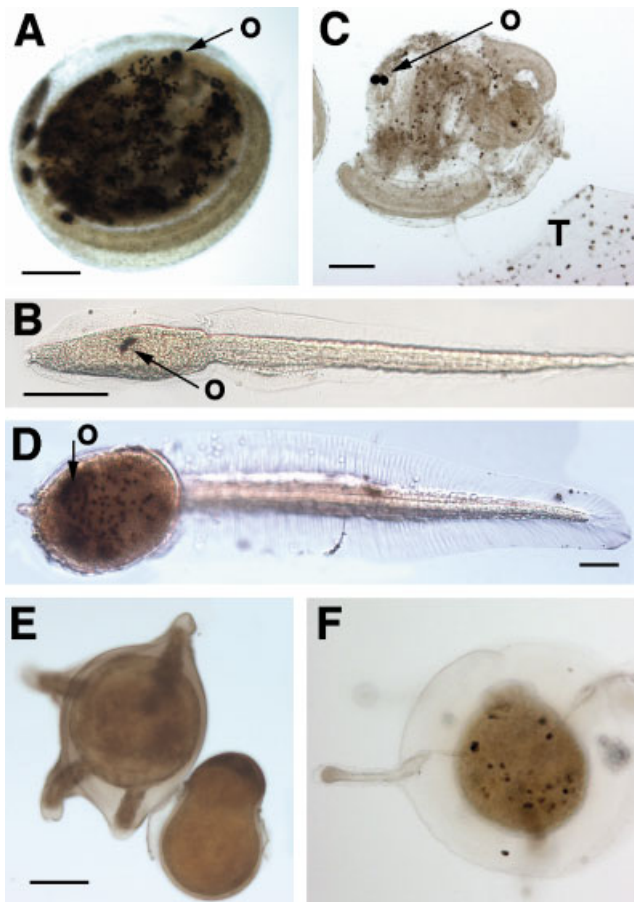


Fig. 2. BTPC: (A) a *Morchelium* tailbud embryo showing BTPC in the trunk; (B, C) a *Ciona* tadpole lacking BTPC (B) and 7-day juvenile with BTPC in body and tunic (T) cells (C); the juvenile was dissected from the tunic (shown on lower right) after DOPA oxidase assay; (D) a *Botryllus* swimming larva containing BTPC in the trunk; (E, F) *M. occulta* hatching larva (E, right) and 1-day juvenile (E, left) lacking BTPC and 2.5-day juvenile with BTPC (F). o: Melanized ocellus and otolith. In (A, B, D) anterior is on the left. Scale bars are 100 μ m in (A) and (D) and 50 μ m in (B), (C), and (E) (magnification is the same in E and F).

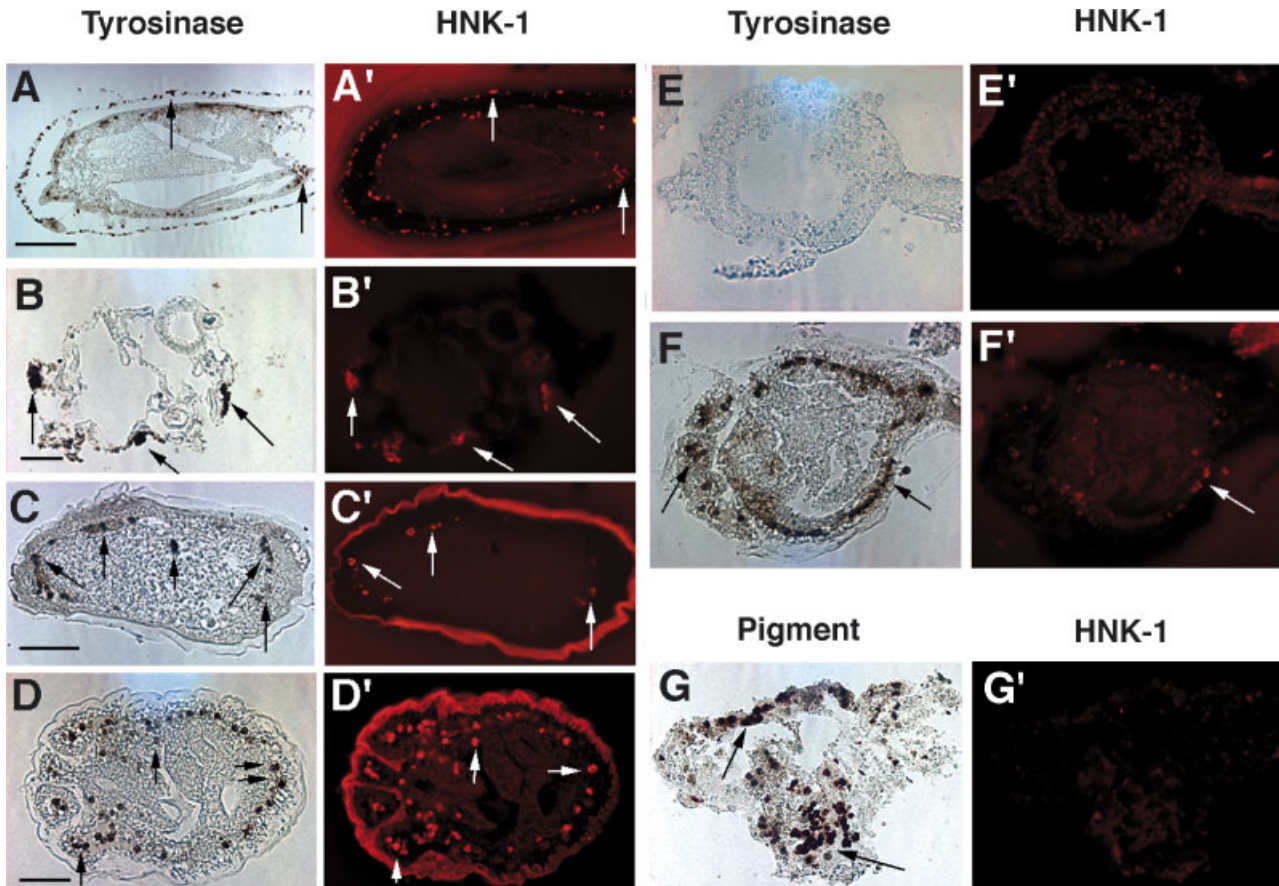


Fig. 3. Double detection of HNK-1 and tyrosinase activity or pigmentation in the same specimen. (A–G) Bright field microscopy showing BTPC (A–F) or pigment (G) distribution in sections. Black arrows depict BTPC (A–F) or pigment cells (G). (A'–G') Fluorescence microscopy showing HNK-1 expressing cells. White arrows depict HNK-1 positive BTPC. (A, A') *Morchelium* swimming tadpole; (B, B') a *Ciona* 7-day juvenile; (C, C') an *M. citrina* tailbud stage embryo; (D, D') a *Botryllus* black morph early swimming tadpole; (E, E') a *Botryllus* albino morph early swimming tadpole; (F, F') a *Botryllus* black morph late swimming tadpole; (G, G') a *Botryllus* black morph metamorphosed juvenile. In (A), (A') and (C)–(F') anterior is on the left. Scale bars are 100 μm in (A) and (D) (magnification is the same in D–G) and 50 μm in (B) and (C).

The relative numbers of BTPC and HNK-1 positive cells were followed by double detection experiments during *Botryllus* development. In late tailbud stage embryos (data not shown) and young tadpoles (Fig. 3D and D'), most BTPC were HNK-1 positive. As tadpoles matured and began to settle prior to metamorphosis, the number of HNK-1 negative BTPC increased, particularly in the anterior region of the trunk where pigment cell differentiation is initiated (Fig. 3F and F'). In post-metamorphic juveniles HNK-1 and tyrosinase expression ceased following pigment cell differentiation (Fig. 3G and G'). The results are consistent with a precursor–product relationship between HNK-1 positive cells, BTPC, and body pigment cells.

Natural *Botryllus* color morphs (Bancroft, '03; Waterson, '45; Milkman, '67) provide another way

to test the relationship between HNK-1 expression, BTPC, and body pigment cells. Different *Botryllus* colonies show blue/black pigment cells, red/orange pigment cells, light reflecting (white) pigment cells, or different combinations of these chromophores. Dominant or recessive genes inherited in a simple Mendelian fashion control the presence or absence of specific chromophores (Sabbadin, '82). We examined HNK-1 and tyrosinase expression in black pigment morphs, which have melanin-containing pigment cells distributed throughout the zooid body and light-reflecting chromophores concentrated in double intersiphonal bands (Fig. 4A), and in albino morphs, which lack body and inter-siphonal band pigment cells (Fig. 4B).

The results showed that the tailbud embryos and tadpoles obtained from black morph colonies

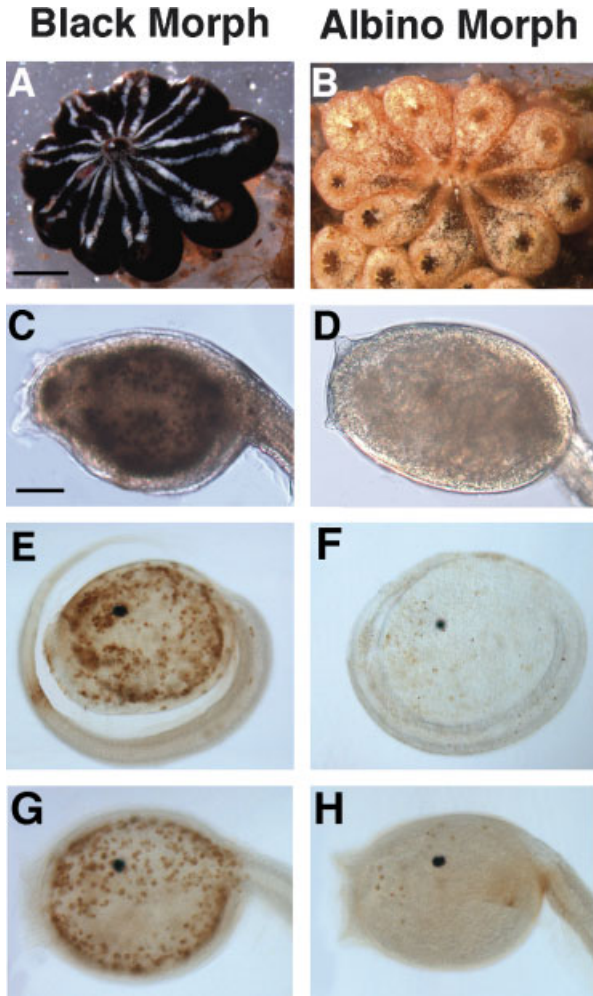


Fig. 4. BTPC and HNK-1 expression in *Botryllus* black (A, C, E, G) and albino (B, D, F, H) morphs. (A, B) Black (A) and albino (B) colonies; (C, D) BTPC in swimming larvae; (E–H) HNK-1 expression in tailbud embryos (E, F) and swimming larvae (G, H). In (C)–(H) anterior is on the left. Scale bar in (A) is 1 mm (magnification is the same in A, B) and 100 μ m in (C) (magnification is the same in C–H).

had many BTPC (Fig. 4C) and HNK-1 positive cells (Fig. 4E and G), whereas those obtained from most albino morph colonies lacked BTPC (Fig. 4D) and were also HNK-1 negative, with the exception of a few cells with faint HNK-1 expression (Fig. 4F and H). The absence of HNK-1 and tyrosinase expression was confirmed in sections of albino tadpoles by double labeling (see Fig. 3E and E'). However, a few albino morph colonies contained a mixed population of tadpoles, some with numerous BTPC and HNK-1 positive cells and others devoid of tyrosinase or HNK-1 expression. The latter results are attributed to heterozygosity: albinism is dominant with respect to black

pigmentation (Sabbadin, '82). However, we saw no cases in which black pigment or albino *Botryllus* tadpoles contained BTPC but lacked HNK-1 expression or vice versa.

DISCUSSION

In this study, we have addressed some key issues concerning the evolution and development of ascidian neural crest-like cells. First, did neural crest-like cells evolve in only a few species, perhaps those with structurally complex larvae, or are they ubiquitous in ascidians? Second, are neural crest-like cells responsible for body pigmentation in diverse ascidians? We surveyed a wide range of ascidian species for cells expressing HNK-1 antigen and tyrosinase to address these questions. We have shown that neural crest-like cells are present in diverse ascidian species and develop into pigment cells or their precursors.

Neural crest-like cells in ascidians

Based on HNK-1 expression, neural crest-like cells were detected in every ascidian species we surveyed, including those with solitary and colonial organizations, with and without adulation, with small or large larvae, and with tailed and tailless larvae. Thus, our survey suggests that this cell type is widespread, if not ubiquitous, in ascidians. Three groups of tunic-bearing animals, the ascidians, larvaceans, and thaliaceans, are traditionally classified as tunicates (or urochordates) (Jeffery and Swalla, '97). Whether the non-ascidian tunicates also exhibit neural crest-like cells is currently unknown. However, recent molecular phylogenies place the larvaceans and thaliaceans within aplousobranch and phlebobranch ascidian groups, respectively (Wada, '98; Swalla et al., 2000; Stach and Turbeville, 2002), suggesting that our sampling of species across the three ascidian suborders may be sufficient to be representative of tunicates. Thus, we conclude that neural crest-like cells did not evolve independently in a particular ascidian group but instead were a primitive feature of the ancestral ascidian or tunicate.

We usually found HNK-1 expressing cells scattered within the trunk mesenchyme or in a few species embedded in the larval or juvenile tunic. Ascidian tunic cells are derived from wandering mesenchyme cells that enter the tunic, which lies outside the body wall, by squeezing through the epidermis (Berrill, '50; Cloney and Grimm, '70).

Thus, we assume that HNK-1 positive tunic cells are derived from invasive neural crest-like cells. We observed HNK-1 expression in neural crest-like cells as early as the late tailbud stage in some species. However, the embryonic origin of these cells could not be determined because when first detected they were already dispersed throughout the larval trunk or juvenile body. Furthermore, the viviparous species we have examined brood embryos, and embryogenesis usually arrests after their removal from the parent (Berrill, '35), complicating analysis of early development. Nevertheless, in *Ciona*, an ovoviparous species, HNK-1 positive cells were initially observed adjacent to the developing neural tube, suggesting a dorsal origin of neural crest-like cells, as previously shown by DiI labeling in *Ecteinascidia* (Jeffery et al., 2004). Future studies will examine the embryonic origins of neural crest-like cells in *Ciona*, in which all developmental stages are accessible and cell lineages are known with certainty.

In vertebrate neural crest cells, HNK-1 antigen is expressed during migration but ceases after migrating cells reach their destinations and begin to differentiate (Hall, '99; Le Douarin and Kalcheim, '99). Although direct evidence is available for migration of ascidian neural crest-like cells from previous studies with *Ecteinascidia* (Jeffery et al., 2004), cell tracing was not practical in other species examined here because of the small size of embryos or the inability of brooded embryos to develop outside the parent. Nonetheless, in the species we have studied neural crest-like cells show more restricted distributions at early stages compared to later stages of development, which is consistent with migration, and their likely invasion of the tunic suggests that they are indeed engaged in migratory activity. Furthermore, the loss of HNK-1 expression after the appearance of pigment cells suggests that HNK-1 downregulation may be correlated with the beginning of cell differentiation. Thus, similar to vertebrate neural crest cells, HNK-1 expression in ascidian neural crest-like cells may be restricted to a migratory phase prior to cell differentiation.

Neural crest-like cells, larval complexity, and adult development

The complexity of ascidian larval morphology is determined by the extent of adulation: the precocious development of adult features in the larval trunk (Berrill, '35; Jeffery and Swalla, '92).

The existence of species with adulation in each of the three ascidian suborders implies that this was either the primitive developmental mode in the ascidian ancestor or that it evolved independently several times in diverse ascidians. Adulation can be excessive with many adult structures beginning to differentiate in the tailbud embryo, or minimal with little or no differentiation of adult structures until after metamorphosis.

The temporal differences in HNK-1 expression between various ascidian species are strongly correlated with the extent of larval structural complexity and adulation. In three aplousobranch species, the phlebobranch *Ecteinascidia* (Jeffery et al., 2004), and the stolidobranchs *Botryllus*, *Botrylloides*, and *M. citrina*, which are characterized by adulation, HNK-1 expression begins during larval development, when the siphons, branchial sac, endostyle, digestive tract, and heart start to differentiate. In contrast, HNK-1 expression does not begin until after larval hatching in the phlebobranch *Ciona*, whose tadpoles show only a few adult primordia. Similarly, in stolidobranchs lacking adulation, which have simple larvae that are virtually devoid of adult primordia, HNK-1 antigen does not appear until after metamorphosis, when adult tissues and organs begin to differentiate. Thus, it is likely that ascidian neural crest-like cells are involved in adult development. As discussed below, most neural crest-like cells appear to be body pigment cells precursors, whose differentiation is part of the adult developmental program.

We also found that the number of HNK-1 expressing cells can vary in different ascidian species. Accordingly, species with large complex larvae usually have more of these cells than those with small simple larvae at approximately the same developmental stage. There are two explanations for differences in the abundance of neural crest-like cells. First, species with complex larvae develop very slowly (Berrill, '35) and more neural crest-like cells may accumulate during prolonged larval development. Second, there could be a specific reason for increasing the number of HNK-1 positive cells in species with complex larvae. For instance, more neural crest-like cells may be required in highly pigmented species, such as *Ecteinascidia*, *Botryllus*, and *Botrylloides*, than in mostly colorless species, such as *Ciona*. Our data are consistent with the second hypothesis. If the number of HNK-1 positive cells can vary in relation to pigmentation, then it is possible that these cells could be lost or modified

in albinistic species, as we have observed in albino morphs of *B. schlosseri*.

Neural crest-like cells and pigmentation

Many ascidian species exhibit pigment cells distributed throughout the body and/or concentrated in the siphons and inter-siphonal regions. Prime examples are *Ecteinascidia*, which has orange pigment cells (Jeffery et al., 2004), and *Botryllus*, which is characterized by different color morphs and types of pigment cells (Waterson, '45; Milkman, '67; Sabaddin, '82; Bancroft, 2003). Other ascidian species, such as *Ciona*, have colorless larvae and pale adults, but a small number of pigment cells are formed in juveniles, particularly around the openings of the siphon (Chiba et al., 2004). Although little is known about the embryonic origin of body pigment cells in ascidians, they are often designated, perhaps misleadingly, as a type of blood or coelomic cell. Many different cell types are found in ascidian circulating blood and their functions and lineage relationships are not completely understood (Wright, '81; Satoh, '94). However, pigment cells can be distinguished from all freely circulating blood cells in that most of them are firmly embedded within body tissues, such as the body wall, branchial sac, and siphons.

Genome-wide searches in *C. intestinalis* have uncovered orthologous genes coding for enzymes involved in multiple pigment-forming biochemical pathways in vertebrates, including the conversion of L-tyrosine and L-DOPA into melanin catalyzed by tyrosinase (Takeuchi et al., 2005). Swalla and Jeffery ('91) described tyrosinase expression in cells throughout the body (BTPC) during juvenile development in the ascidian *Bostrichobranchnus digonas*. These findings, along with the well-known role of vertebrate neural crest cells in generating pigmentation (Hall, '99; Le Douarin and Kalcheim, '99), were an impetus to investigate BTPC in diverse ascidian species and their relationship to neural crest-like cells. BTPC were observed in each of the surveyed ascidian species, including those with intense pigmentation in the larva and/or zooid (*Ecteinascidia*, *Botrylloides*, *Botryllus*) and those with more localized pigmentation appearing in juveniles (*Ciona*).

Several lines of evidence suggest that there is a close relationship between HNK-1 positive cells, BTPC, and body pigment cells. First, double labeling showed that most BTPC express HNK-1, suggesting that they may be equivalent cell types. Second, in *Ciona* HNK-1 expression appears

before tyrosinase, and in *Botryllus* BTPC are still present as HNK-1 expression is declining prior to the final steps of pigment cell differentiation. These observations are consistent with a precursor-product relationship between HNK-1 positive cells, BTPC, and pigment cells, respectively. Third, *Botryllus* homozygous albino morphs lacking pigment cells do not exhibit BTPC or show HNK-1 expression. Indeed, the inability to detect HNK-1 antigen or BTPC in albino morph tadpoles suggests that body pigmentation may be the only developmental role of neural crest-like cells, at least in *Botryllus*. Even HNK-1 positive tunic cells show tyrosinase activity, and therefore may represent sites of melanin pigment deposition outside the body. However, we cannot exclude the possibility that some HNK-1 positive cells may have additional fates, either in *Botryllus* or other species, because HNK-1 antigen could disappear from the cell surface rapidly during differentiation, leaving some non-pigment cell derivatives undetectable by our methods. Further analysis of the derivatives of ascidian neural crest-like cells will require indelible labeling to precisely follow their lineage during development.

Relationship to vertebrate neural crest cells

One of the reasons for examining neural crest-like cells in different ascidian species was to assess their relationship to vertebrate neural crest cells. In various ascidian species, HNK-1 expression was restricted to a class of mesenchymal cells whose primary role is to generate body pigmentation. In vertebrates, all body pigment cells, including melanophores, iridophores, and xanthophores, are neural crest derivatives (Erickson, '93). Thus, the most striking similarity between vertebrate neural crest cells and ascidian neural crest-like cells is their mutual role in body pigment cell development. The origin of neural crest-like cells near the dorsal neural tube, migratory activity, and association with siphon primordia (Jeffery et al., 2004), probable ascidian placode homologues (Mazet and Shimeld, 2005), also imply a close relationship between ascidian neural crest-like cells and vertebrate neural crest cells. Based on these similarities, we propose that the ascidian and vertebrate cells are homologous and had a common origin during chordate evolution. Evidence has recently been obtained for a closer evolutionary relationship between tunicates and

vertebrates than previously suspected (Delsuc et al., 2006).

Despite their resemblances, ascidian neural crest-like cells are distinct in several ways from their presumed vertebrate homologues. First, ascidian neural crest-like cells migrate singly rather than in streams. Second, ascidian neural crest-like cells are not produced along the entire neuroaxis: they are excluded from the tail, when it is present. Third, and most importantly, ascidian neural crest-like cells probably have much more restricted developmental fates than vertebrate neural crest cells. Presumably, primordial neural crest-like cells maintained their primitive function, the generation of body pigmentation, in ascidians, whereas they evolved many new functions in vertebrates, perhaps by cooption of gene regulatory networks (Meulemans and Bronner-Fraser, 2005).

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