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Review

Chordate ancestry of the neural crest: New insights from ascidians

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Abstract

This article reviews new insights from ascidians on the ancestry of vertebrate neural crest (NC) cells. Ascidians have neural crest-like cells (NCLC), which migrate from the dorsal midline, express some of the typical NC markers, and develop into body pigment cells. These characters suggest that primordial NC cells were already present in the common ancestor of the vertebrates and urochordates, which have been recently inferred as sister groups. The primitive role of NCLC may have been in pigment cell dispersal and development. Later, additional functions may have appeared in the vertebrate lineage, resulting in the evolution of definitive NC cells.

Keywords: Neural crest; Neural crest-like cells; Vertebrates; Ascidians; Pigment cells

Contents

1. Introduction: evolution of the neural crest

The complexity of the vertebrate body is due in large part to the neural crest (NC), a source of multipotent mesenchymal cells originating at the border of the neural and non-neural ectoderm [1–4]. During neural tube formation, NC cells undergo an epithelial to mesenchymal transformation and subsequently migrate through distinct pathways into the interior and periphery of the embryo. In most vertebrates, NC cells delaminate and migrate in a temporal sequence along the neuroaxis, beginning at the level of the presumptive diencephalon. Cranial NC cells stream between the rhombomeres of the hindbrain and enter the pharyngeal pouches and other tissues of the head. Trunk NC cells migrate between the neural tube and somites, within the sclerotome, or between the dermal and epidermal layers. After NC cells reach their final destinations, they differentiate into a myriad of different cell and tissue types, such as sensory neurons, the craniofacial and pharyngeal arch skeletons, the peripheral nervous system, smooth muscle, calcitonin-producing cells of the ultimobranchial gland, adrenal endocrine cells, and all body pigment cells (melanophores, xanthophores and iridophores) [5].

The phylum Chordata consists of three extant groups: urochordates (or tunicates), cephalochordates (amphioxus), and vertebrates, which are unified by having a dorsal CNS, a notochord, pharyngeal gill slits, and a post-anal tail at some stage in their life cycle. The existence of NC cells in jawless vertebrates [6,7] and possibly in early vertebrate fossils, such as

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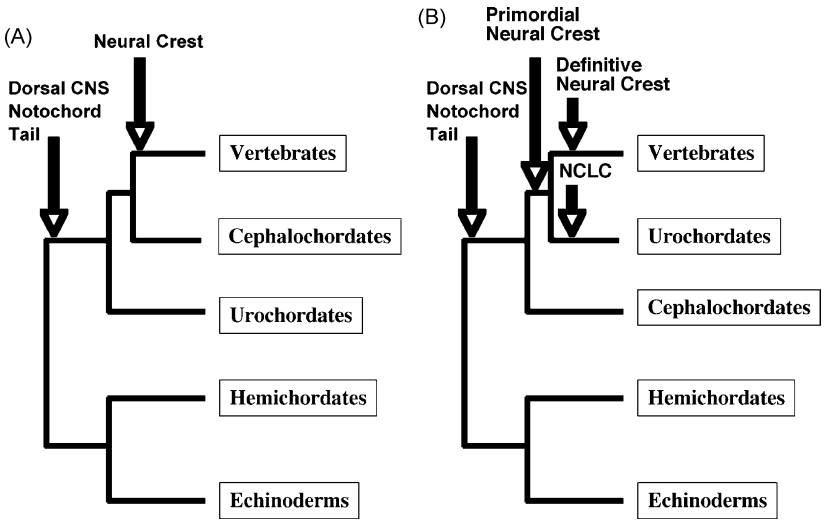

Fig. 1. NC evolution. (A) Traditional view. (B) View incorporating new insights from ascidians. Alternative deuterostome trees are based on (A) rDNA sequences, by which cephalochordates are the closest living relatives of vertebrates [23,24], and (B) metagene phylogenies [19–22] suggesting that urochordates are the sister group of vertebrates. NCLC: neural crest-like cells.

Myllokunmingia [8], implies that the NC had already evolved at the base of the vertebrate radiation. Because cells migrating from the dorsal neural tube and major derivatives attributed to the NC are thought to be absent in invertebrate chordates, it has been widely accepted that NC cells were a vertebrate invention [9,10] (Fig. 1A). Furthermore, the presence of NC cells in basal vertebrates suggests that they could have been a key factor in vertebrate evolution [9]. However, invertebrate chordates do have some features resembling vertebrate NC derivatives: pharyngeal calcitonin-like cells [11], migratory neurogenic cells [12,13], cartilage-like tissues [14], body pigment cells [15], and peripheral sensory neurons [16]. These features provide clues that NC cells may not be restricted to vertebrates [17,18]. Thus, it is still unclear whether NC cells suddenly appeared *de novo* in a primitive vertebrate, or latent neural crest-like cells (NCLC)[1] were already present in an ancestral chordate and evolved the unique derivatives of bonafide NC cells after vertebrates diverged from the other chordate lineages.

Urochordates are a prime group to probe NC origins because recent molecular phylogenies infer them as the sister group of the vertebrates (Fig. 1B) [19–22], contrary to traditional views placing cephalochordates as the closest living vertebrate relatives (Fig. 1A) [23,24]. In this article, we explore the problem of NC ancestry by reviewing new evidence for NCLC in ascidians, one of the three groups of urochordates. We conclude that NCLC were already present in the common ancestor of the urochordates and vertebrates (Fig. 1B).

2. Early searches for neural crest in invertebrate chordates

Vertebrate NC cells are defined by the following criteria: (1) embryonic origin between neural and non-neural ectoderm, (2) delamination and extensive migration from the dorsal neural tube, (3) characteristic gene expression profiles, and (4) multipotency [25]. Early searches for NC cells in amphioxus and several ascidian species focused on the first and third criteria, seeking cells along the border of the neural plate that express a profile of genes in common with vertebrate NC cells [10,18,25–28]. These studies identified cells that express a few of the important NC-related genes, such as *msx*, *snail/slug*, and *pax*3–7 [16,29–35], however, they did not appear to undergo an epithelial to mesenchymal transformation or migrate extensively. Thus it was concluded that these cells were not definitive NC cells [18,25,26].

The ontogeny and behavior of vertebrate NC cells, which first become easily observable as they begin to migrate during neurulation, biased the early searches for NCLC in other chordates. Thus in the previous studies, NC cells were sought only around the time of neurulation, and not at later stages of development. Moreover, the ascidian species that were investigated in these studies (*Molgula oculata*, *Halocynthia roretzi*, and *Ciona intestinalis*) all have relatively small, streamlined, and possibly structurally reduced larvae (see below). Their small size discouraged classic approaches, such as cell marking, which were instrumental in demonstrating the migratory activity and multipotency of vertebrate NC cells [1–3].

3. Adultation and the complexity of ascidian larvae

Ascidians have a life cycle consisting of a dispersal phase, in which a motile tailed larva (tadpole) forms during embryonic development, and a subsequent sessile phase, in which a filter-feeding adult develops after metamorphosis [36,37]. Metamorphosis involves retraction of the larval tail into the head, degradation of some larval tissues, and differentiation and morphogenesis of adult tissues and organs. Some ascidians, primarily members of the family Molgulidae (for example *Molgula occulta*), have secondarily lost the tailed larva, proceeding to adulthood by direct development or metamorphosis of a tailless

[1] In this article, NCLC is used to designate cells that resemble vertebrate neural crest cells in some of their features, whereas NC is used to refer to definitive neural crest cells in vertebrates.

larva [38]. As described above, early searches for ascidian NCLC were restricted to *M. oculata* (which exhibits a tailed larva), *Ciona*, and *Halocynthia*. Although frequently studied in laboratories because of their short developmental timetables and other attributes, the larvae of these animals are small and simplified. In contrast, many other ascidian species have large, structurally complex larvae [39,40]. The complexity of larval morphology is a consequence of adultation: the precocious differentiation of adult tissues and organs in the larval head during embryonic development [41].

In this article, ascidians are separated into four categories based on the extent of adultation and pre-development of adult structures (Fig. 2). Category A shows essentially no adultation, as exemplified by the tailless species *M. occulta*, although most ascidians in this category have a tailed larva (Fig. 2A). Category B shows minimal complexity but some pre-development of adult structures, including siphon primordia, a branchial sac perforated by a few gill slits, and an endostyle (the ascidian thyroid homologue [43]), as in *Ciona* (Fig. 2B). Category C shows more extensive adultation, with more highly developed siphon primordia and more branchial gill slits, as in *Botryllus schlosseri* (Fig. 2C). Finally, Category D shows extreme adultation (Fig. 2D), in which more adult organs, including a gastrointestinal tract and a well developed heart, have already appeared during early larval development. The prime Category D example is *Ecteinascidia turbinata* (Fig. 2D). There are also differences in larval size and rate of development among the four categories. Category A generally has the smallest, and Category D the largest larvae. The 'giant' *Ecteinascidia* tadpole, which approaches the size of a smaller amphibian tadpole, requires about 10–15 days to develop from egg to hatching, compared to 12 or 18 h in most *Molgula* species or *Ciona intestinalis* respectively at comparable temperatures. Examples of species with adultation are found within each of the three ascidian suborders: Aplousobranchia, Phlebobranchia, and Stolidobranchia.

The evolutionary history of adultation has not been completely resolved in ascidians [44,45]. Accordingly, it is difficult to predict whether larval complexity has generally increased (e.g. Categories A and B being primitive and Categories C and D derived) or has decreased and is regressive. Once again, we emphasize that early searches for NCLC were conducted exclusively in species with Category A and B larvae. Thus, if the simplicity of these larvae is derived and regressive then it is possible that migratory cells were present in basal ascidians and lost during subsequent tadpole streamlining. This possibility served as the impetus for examining the existence of NCLC in ascidian species with larger and more complex larvae that those in Categories A and B.

4. Migratory neural crest-like cells in *Ecteinascidia*

Ecteinascidia, which has a "giant" tadpole, was the first ascidian species used to explore the existence of migratory NCLC using classical approaches [46]. To determine whether migratory

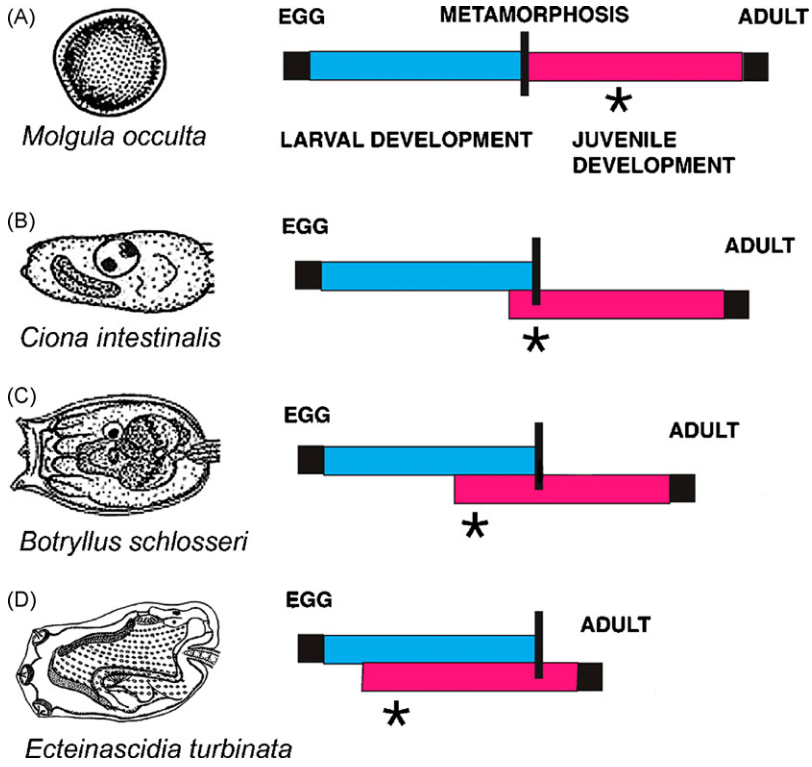

Fig. 2. Adultation and complexity of ascidian larvae. Category A: Simplified larvae with no adultation as in *Molgula occulta*. Category B: Minimal adultation as in *Ciona intestinalis*. Category C: Moderate adultation as in *Botryllus schlosseri*. Category D: Extreme adultation as in *Ecteinascidia turbinata*. (A–D) Left: structure of larval heads. Right: line diagrams illustrating the extent of overlap in larval (blue) and juvenile adult (red) development. Vertical line: metamorphosis. Asterisks: Approximate timing of the beginning of HNK-1 expression. Drawings of larvae based on Jeffery [42] (A) and Berrill [39] (B–D).

cells are generated at the dorsal midline, the vital lipophilic dye DiI was injected into the *Ecteinascidia* neural tube near the otolith and ocellus, melanin-containing sensory organs that mark the neural primordium. As shown in Fig. 3A–F, DiI-labeled cells migrated from the dorsal midline into the siphon primordia, the branchial sac, and the body wall. Migration was first detected near the anterior CNS during the mid tailbud stage, later spreading to the posterior region of the head, and was still observed in the swimming larva. The DiI labeled cells migrate singly rather than in streams. During migration they are morphologically distinct from any other cell in the larva [46]. Three migratory pathways were detected (Fig. 3G–J): (1) anterior toward the oral siphon primordium, (2) posterior toward the anal siphon primordium, and (3) ventral into the larger part of the head and future adult body, which contains the branchial sac and associated tissues. Migration into the siphon primordia is noteworthy because they have been proposed to be the homologues of vertebrate placodes [10,17,47,48], which sometimes collaborate with NC cells to form sensory organs [2,3]. Furthermore, ventral migration into the branchial sac, the likely homologue of vertebrate pharyngeal gill pouches [49], resembles the migratory pathway of cranial NC cells [1–3]. In addition, some of the DiI-labeled cells crossed the epidermis and continued to migrate within the developing tunic. Migratory cells do not enter the tail proper, although a few were observed in the surrounding tunic, which migrated from the larval head region. These results represent the first evidence of migratory NCLC cells in an invertebrate chordate.

Two markers of vertebrate NC were used to further investigate the possibility that the migratory cells are NCLC [46]. Cells similar in morphology and spatial position to those labeled with DiI, were stained with a monoclonal antibody to the HNK-1 glycoprotein antigen (Fig. 4A and D). In vertebrate embryos, the HNK-1 antibody recognizes NC cells during their migratory phase, as well as a few other non-NC derived tissues and cells during later development [2,3,50–53]. However, in *Ecteinascidia*, HNK-1 antibody recognized only a single cell type, which exhibited the same morphology and spatial position as the DiI labeled migratory cells. Cells resembling the DiI labeled migratory cells also expressed a second NC marker, a homologue of the vertebrate *zic2* gene (*Etzic*), (Fig. 4B and E). The *zic2* gene is critical in regulating vertebrate NC development and can induce unspecified ectoderm to develop into migratory NC cells [54–56]. Therefore, the migratory cells identified by DiI labeling also appear to coincide with cells that express some of the classic vertebrate NC markers.

The *Ecteinascidia* larva is richly pigmented [46,57]. Large orange pigment cells are concentrated in the siphons and distributed throughout the body wall (Fig. 4C) and the internal organs of the head, although they are lacking in the tail. Some of the migratory cells differentiate into these pigment cells [46], as determined by co-localization of DiI labeling and orange pigment granules in the same cells, demonstrating that NCLC contribute to body pigmentation in *Ecteinascidia*. In summary, NCLC originate near the dorsal midline, migrate extensively through at least three distinct pathways, and differentiate into body pigment cells in *Ecteinascidia* embryos.

5. Neural crest-like cells are widespread in ascidians

There are two possible explanations for the presence of NCLC in *Ecteinascidia*. First, migratory cells that express HNK-1 and *Etzic* may have evolved independently by convergence. The migratory activity of these cells could be related to the large size and high complexity of the Class D *Ecteinascidia* tadpole. Alternatively, NCLC could be a general characteristic of ascidians. This possibility would be consistent with a common origin of urochordate NCLC and vertebrate NC cells.

To distinguish between these possibilities, the HNK-1 marker was used to survey NCLC in diverse species distributed among the three ascidian suborders [58]. HNK-1 stained cells were observed in 3 aplousobranchs, 2 phlebobranchs, and 6 stolidobranchs, bringing the total number of species with HNK-1 positive cells (including the phlebobranch *Ecteinascidia*) to 12 (Fig. 5). Fig. 6A–D shows examples of HNK-1 expression in Category A (Fig. 6D; *M. occulta*), Category B (Fig. 6A and B; *Ciona*), and Category C (Fig. 6C; *Botryllus*) larvae. Among the ascidians with HNK-1 positive cells are both solitary and colonial species, species with different developmental modes (including those with tailed or tailless larvae), species with large and small embryos, and species with morphologically simple or complex larvae (Fig. 5). However, the timing of HNK-1 expression, and thus probably cell migration, ranges widely from the embryonic to post-metamorphic stages (see Fig. 2 asterisks). HNK-1 expression begins during the larval tailbud stage in the aplousobranchs *Didemnum*, *Aplidium*, and *Morchellium*, the phlebobranch *Ecteinascidia*, and the stolidobranchs *Botryllus*, *Botrylloides*, and *Molgula citrina*, which are characterized by Category C or D larvae. In contrast, HNK-1 expression does not occur until after larval hatching in the Category B phlebobranch *Ciona* tadpole or after metamorphosis in Category A stolidobranch larvae (*M. occulta*, *Molgula socialis*, and *Styela clava*). If HNK-1 expression is congruent with migratory activity in these ascidians, as it is in vertebrates and apparently in *Ecteinascidia*, then migration appears to be correlated with the beginning of adult rather than larval development. The appearance of HNK-1 positive cells after hatching or metamorphosis in species with Category A and B larvae is a likely reason that NCLC were not recognized in the earlier searches, which were restricted to the period around neurulation. Based on HNK-1 staining, NCLC appear to migrate at later stages of development than would be anticipated from the behavior of NC cells in vertebrate embryos.

The systematic survey suggests that HNK-1 positive NCLC are widespread, if not ubiquitous, in ascidians. Three groups of tunic bearing animals–the ascidians, larvaceans, and thaliaceans–are classified as urochordates. Whether the larvaceans and thaliaceans also exhibit NCLC is currently unknown. However, recent phylogenies embed these two groups within the ascidians [44,59], suggesting that sampling taxa across the ascidian suborders may be sufficient to be representative of urochordates. If this is the case, then NCLC are likely to be a primitive feature of the ancestral urochordate and could share a common origin with vertebrate NC cells.

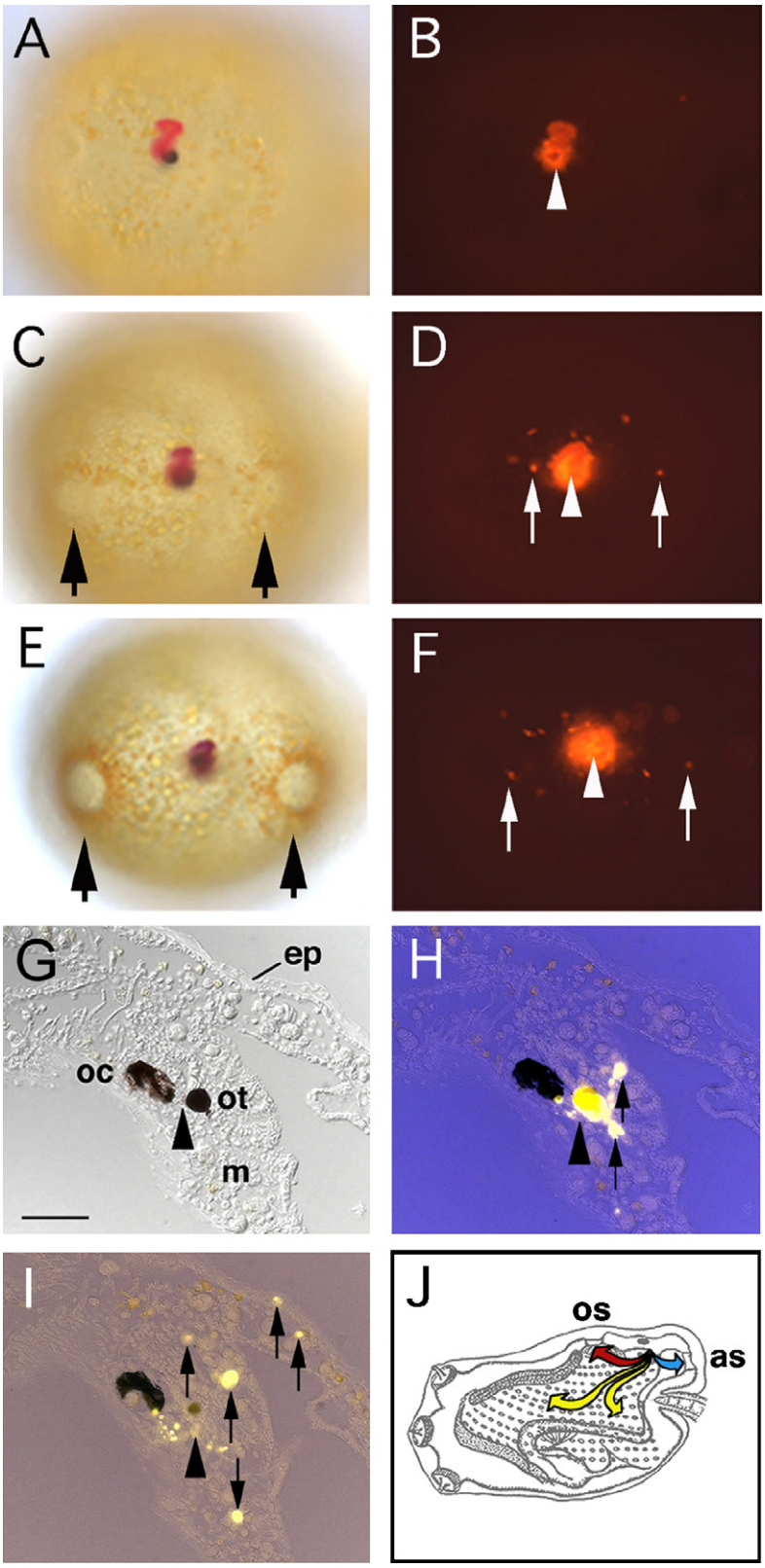

Fig. 3. DiI tracing of migratory NCLC in *Ecteinascidia turbinata*. (A–F) Dorsal views of bright field (left) and fluorescence (right) images of the same mid-tailbud embryo injected in the anterior neural tube near the otolith/ocellus (black dot) at the early tailbud stage. Images shown at 10 min (A and B), 4 h (C and D), and 14 h (E and F) after DiI injection. (G–I) Bright field (G) and fluorescence (H and I) images of a mid tailbud embryo cross serially sectioned with respect to the anterior-posterior axis showing the DiI injection site between the otolith (ot) and ocellus (oc), and DiI labeled cells migrating through the mesoderm (m) and below the epidermis (ep). Scale bar: 30 μm. Arrowheads: DiI injection site. Arrows: DiI labeled cell. (A–I) from Jeffery et al. [46]. (J) Diagram showing the three pathways of cell migration: anterior (red), posterior (blue), and ventral (yellow).

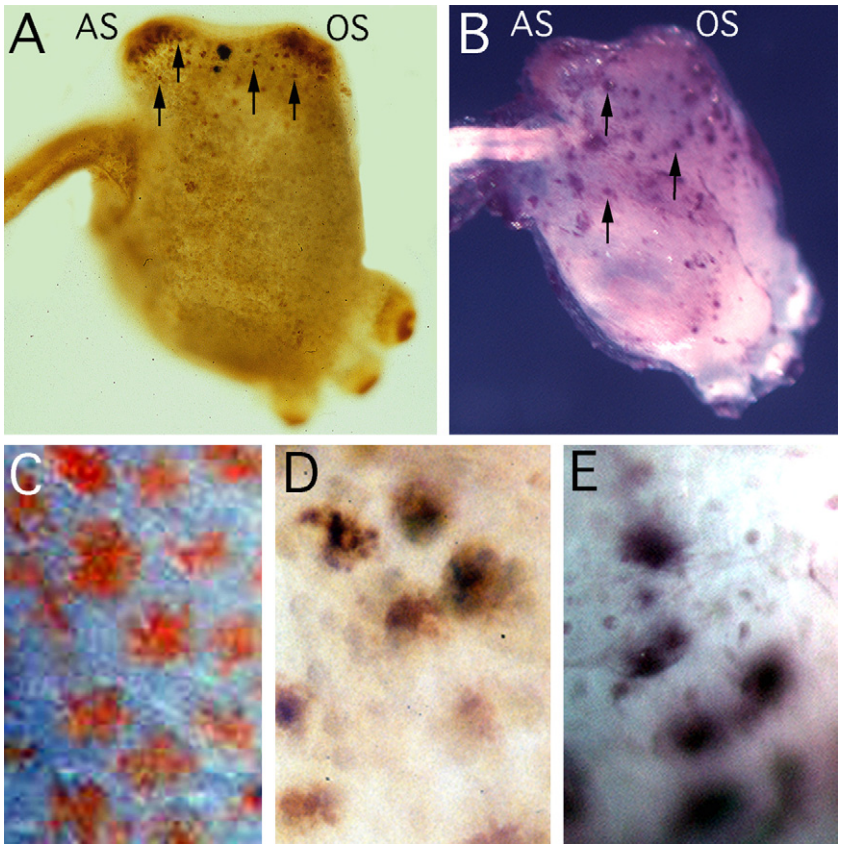

Fig. 4. Expression of NC markers and body pigmentation in *Ecteinascidia turbinata*. (A and B) Lateral views of hatched larvae showing HNK-1 stained (A) and *Etzic* expressing (B) cells (arrows) in the siphons and dorsal body wall. (C–E) Comparison of orange pigment cells (C), HNK-1 stained cells (D), and *Etzic* expressing cells (E) in the larval body wall. OS: oral siphon; AS: anal siphon. From Jeffery et al. [46].

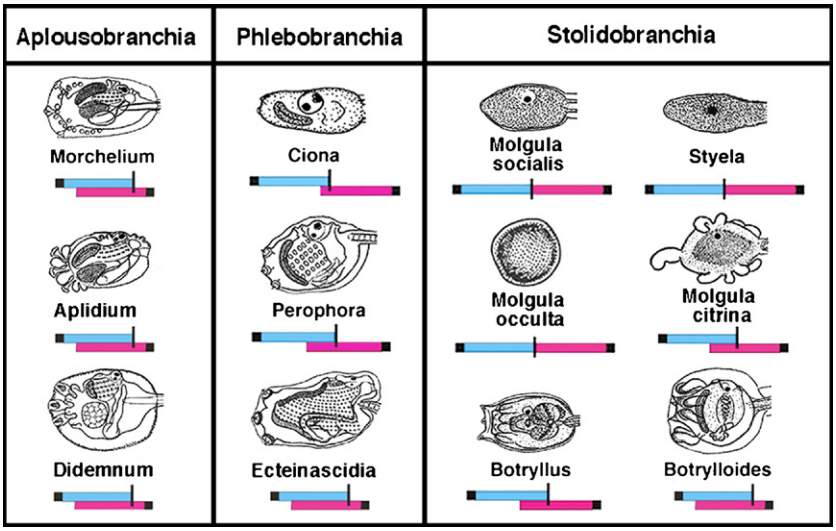

Fig. 5. Aplousobranch, phlebobranch, and stolidobranch ascidians in which HNK-1 positive and BTPC have been demonstrated. Larval heads are shown above line diagrams depicting the extent of adultation (see Fig. 2 for explanation). Larvae are shown with increasing complexity from top to bottom (except in Aplousobranchia). Drawings of larvae based on Jeffery [42] and Berrill [39].

6. Ascidian body pigment cells

Many ascidian larvae and or adults are very colorful, with pigment cells of various hues and reflections distributed throughout the body and/or concentrated in the siphons and inter-siphonal regions. Prime examples are *Ecteinascidia* (Fig. 4C) [46,57] and *Botryllus*, the latter exhibiting many different color morphs with distinctive pigment patterns (see Fig. 7C and D) [60–62]. In contrast, *Ciona* has colorless larvae and pale adults, prompting the Japanese name kata-yurei boya

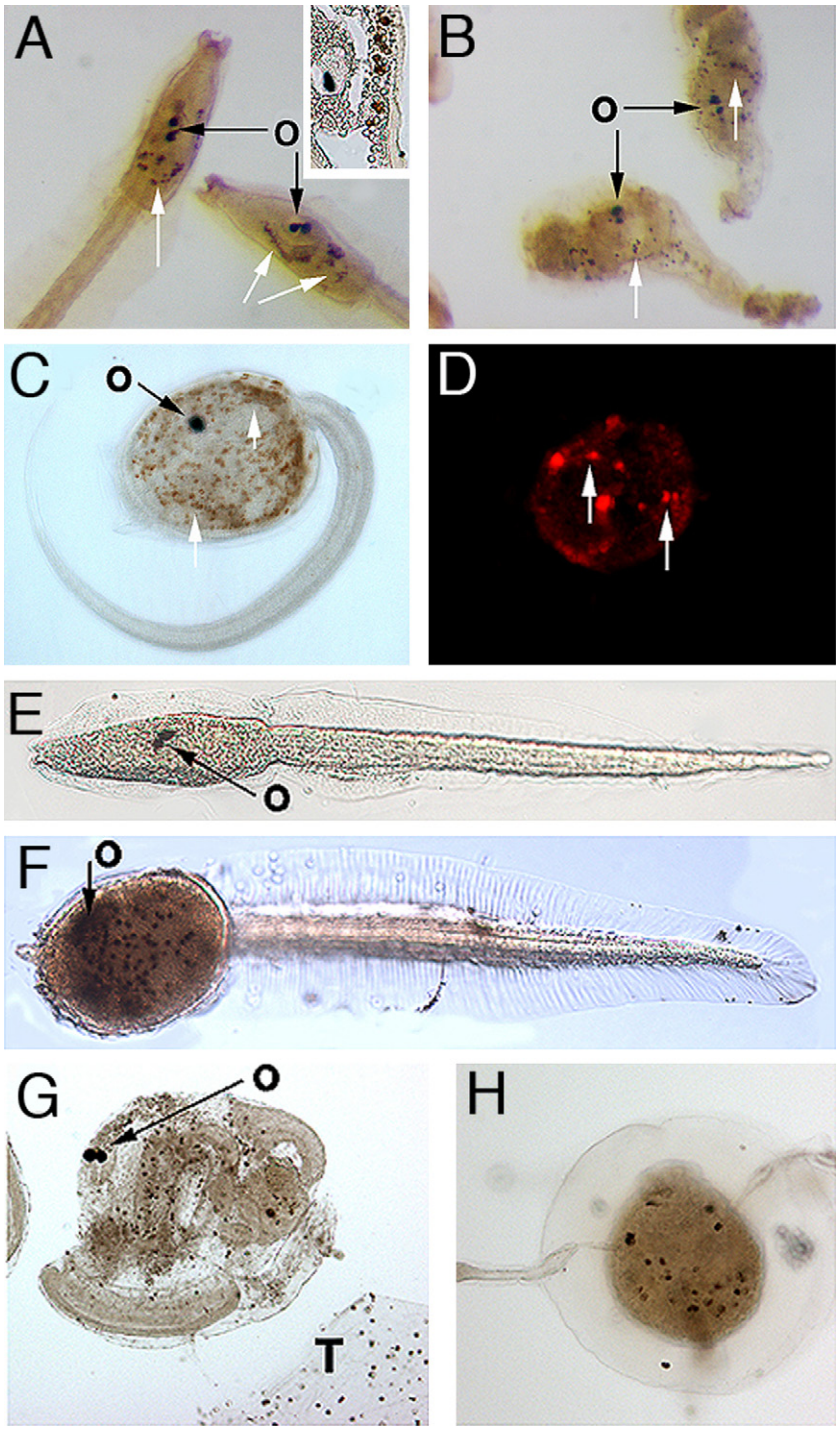

Fig. 6. Examples of HNK-1 positive cells and BTPC in different ascidian species. (A–D) HNK-1 expression. (A and B) *Ciona* (Category B; see Fig. 2) hatched larva (A: inset; high magnification of migrating cells) and during metamorphosis (B). (C) *Botryllus* (Category C) late tailbud embryo. (D) *M. occulta* (Category A) after metamorphosis. White arrows: HNK-1 positive cells. E–H. BPTC. (E and G) *Ciona* (Category B) hatched larva (E), which lacks tyrosinase expression, and young adult (G), which shows tyrosinase expression in the body and in the dissected tunic (T). (F and H) Tyrosinase expression in a *Botryllus* swimming larva (F) and an *M. occulta* after metamorphosis (H). o: otolith and ocellus. From Jeffery [58].

or ghost ascidian. However, pigment cells differentiate after metamorphosis in *Ciona* [63], and in adults are concentrated as a yellow band around the edge of the siphons, which also contains the red-pigmented photoreceptors [64].

Little is known about ascidian body pigment cells, including their embryonic origin, and they are sometimes designated, perhaps misleadingly, as a type of blood or coelomic cell. Many different cell types are found in the circulating blood of ascidians with mostly unknown functions and interrelationships [36,65]. However, pigment cells can be distinguished from freely circulating blood cells because most of them are firmly embedded within the body wall, branchial sac, and siphons.

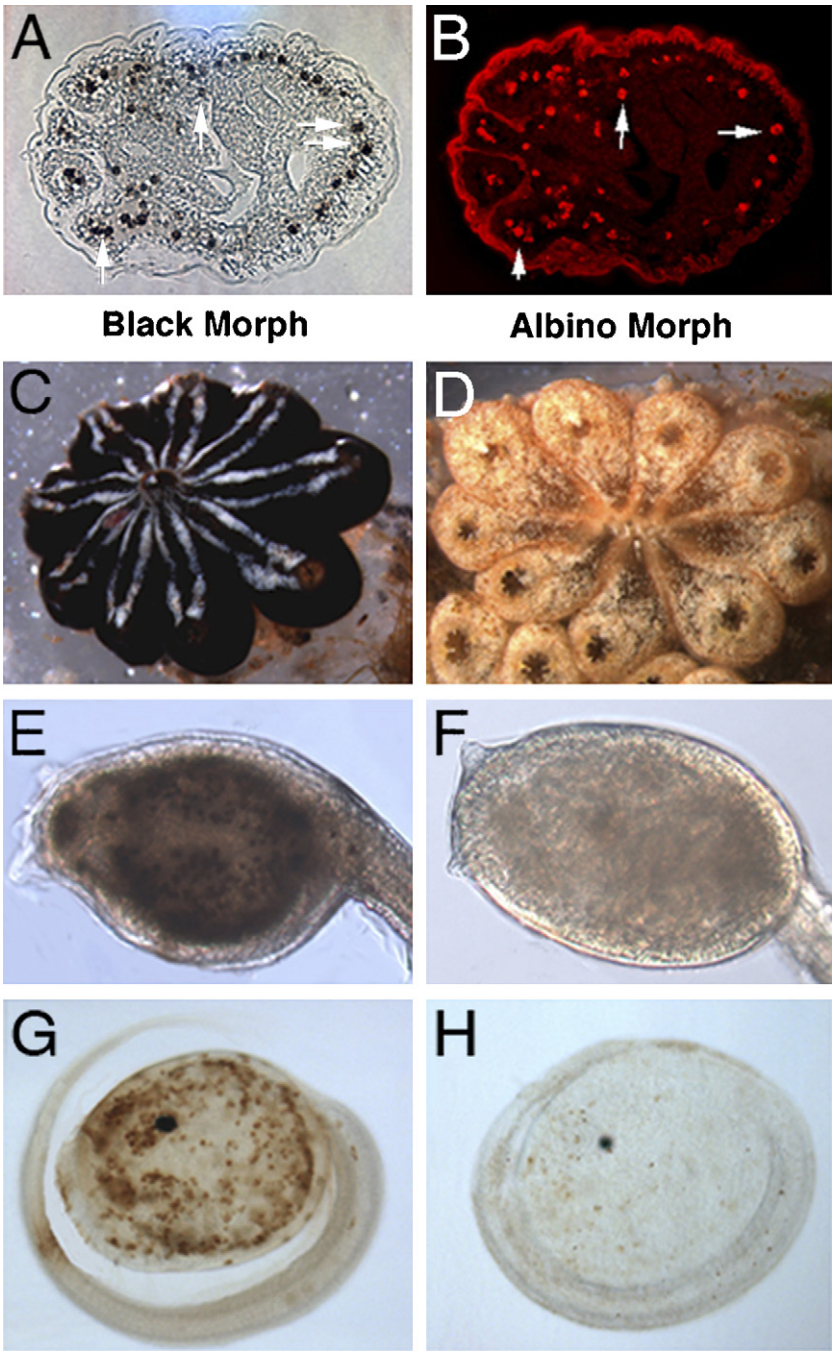

Fig. 7. (A and B) Double detection of tyrosinase (A) and HNK-1 (B) expression in the same *Botryllus* larva. Arrows: BTPC (A) and HNK-1 (B) positive cells. (C and D) Black (C) and albino (D) morph *Botryllus* colonies. (E–H) BTPC (E and F) and HNK-1 expression (G and H) in *Botryllus* black (left) and albino (right) morph larvae (E and F) and late tailbud stage embryos (G and H). From Jeffery [58].

Genome wide searches in *Ciona* have uncovered genes coding for the enzymes involved in multiple pigment-forming biochemical pathways, including the conversion of L-tyrosine and L-DOPA into melanin, which is catalyzed by the key melanogenic enzyme tyrosinase [66]. Swalla and Jeffery [15] described tyrosinase expression in cells distributed throughout the body in young adults of the ascidian *Bostrichobranchus digonas*. Although these cells presumably accumulate melanin, they should not be confused with the melanogenic cells in the otolith and ocellus. The brain pigment cells are embryonic in origin and do not undergo long-range migration, although they move short distances with respect to each other during larval morphogenesis [67–69].

Body tyrosinase positive cells (BTPC) have recently been described in the same diverse ascidian species in which HNK-1 positive cells have been identified (Figs. 5 and 6E–H) [58]. Similar to the HNK-1 stained cells, BTPC were detected in the larval head and tunic but not in the tail in these species. In species with Category A and B larvae, BTPC are undetectable in tailbud stage embryos and tadpoles but appear after metamorphosis

(*Ciona*; Fig. 6E and G; *M. occulta*; Fig. 6H). Species with Category C and D larvae differ in that BTPC are already present in late tailbud embryos and swimming larvae (*Botryllus*; Fig. 6F). Thus, BTPC are present in diverse ascidian species and resemble HNK-1 positive cells in their localization, abundance and timing of tyrosinase expression.

7. Neural crest-like cells and body pigmentation

The taxon survey [58] also indicates that the abundance of HNK-1 stained cells differs markedly among ascidian species at about the same developmental stage. These differences are correlated with the extent of body pigmentation. For example, *Ciona* (Fig. 6A, B, E and G), which lacks intense body pigmentation, exhibits a relatively low number of larval BTPC and HNK-1 positive cells [58]. In contrast, HNK-1 stained cells and BTPC are more numerous in highly pigmented species, such as *Ecteinascidia* (Fig. 4) and *Botryllus* (Fig. 6C and F). This relationship, along with direct evidence showing the differentiation of NCLC in *Ecteinascidia* into pigment cells [46], suggests that body pigmentation may be a major derivative of ascidian NCLC.

Do HNK-1 positive cells differentiate exclusively into body pigment cells? This question was addressed in two different ways [58]. First, procedures were developed to detect HNK-1 and tyrosinase in the same cells. Double labeling experiments in several ascidian species, including *Botryllus* (Fig. 7A and B), showed that most BTPC express HNK-1, indicating that NCLC and BTPC are for the most part equivalent cell types. Second, HNK-1 and tyrosinase expression were compared in *Botryllus* colored and albino morphs. The colonial ascidian *Botryllus* shows many different pigment cells distributed throughout the body and in the inter-siphonal bands [60–62]. Black pigment morphs have melanin-containing pigment cells (Fig. 7C), while albino morphs lack body pigmentation (Fig. 7D). Tailbud embryos and tadpoles obtained from black colonies have many BTPC (Fig. 7E) and HNK-1 positive cells (Fig. 7G), whereas those from albino colonies lack BTPC (Fig. 7F) and contain very few, faintly-stained HNK-1 positive cells (Fig. 7H). The matching of almost every BTPC with an HNK-1 positive cell in double labeling studies and the inability to detect BTPC or appreciable HNK-1 staining in albino tadpoles imply that pigment cell formation could be the only role of these cells in *Botryllus*. In the absence of direct cell tracing studies, however, the possibility that some HNK-1 positive cells may have additional fates cannot be excluded.

8. Ascidian neural crest-like cells and vertebrate neural crest cells: similarities and differences

The most striking similarity between ascidian NCLC and vertebrate NC cells is their common role in pigment cell development. The origins of NCLC near the dorsal neural tube, their migratory activity, and their association with siphon primordia (proposed placode homologues) also suggest a close relationship between ascidian NCLC and vertebrate NC cells. These resemblances support the possibility that the ascidian and vertebrate cells had a common origin during chordate evolution.

Ascidian NCLC are distinct from vertebrate NC cells in several ways. First, ascidian NCLC migrate singly rather than in streams. The absence of segmented blocks of mesoderm and distinct neuromeres in ascidians may partially account for this difference. Second, ascidian NCLC are not formed along the entire neuroaxis; based on the absence of HNK-1 stained cells, they appear to be excluded from the tail. Third, ascidian NCLC migrate at later stages of development than vertebrate NC cells, although avian melanogenic NC cells also show late migration [70]. Fourth, ascidian NCLC may have more restricted fates than vertebrate NC cells, if pigment cell formation is indeed their only function. An intriguing possibility is that body pigmentation may be the primordial role of the ancestral NC [46,71,72], which may have evolved for protection from exposure to ultraviolet radiation in shallow marine habitats. This hypothesis is attractive because it provides a possible adaptive explanation for why the primordial NC attained migratory activity to reach the periphery of the embryo or adult body.

9. Evolution of the neural crest revisited: future directions

The new insights from ascidians described here provide a fresh perspective on the origin and evolution of the NC. They support the possibility that ascidian NCLC and vertebrate NC cells share a common ancestry, perhaps originating from a primordial NC with a primary role of generating body pigment cells (see Fig. 1B). While maintaining their primary function in ascidians as well as in vertebrates, in the vertebrate lineage the primordial NC subsequently evolved many additional fates, perhaps by cooption of gene regulatory networks [73].

Many aspects of ascidian NCLC remain to be defined. The major goals of future studies should be the following. First, the precise embryonic origin and identity of NCLC must be established. Do they originate at the border of neural and non-neural ectoderm, the neural tube itself, or from other sources in the embryo? Is the embryonic origin of HNK-1 positive cells the same in diverse ascidian species? Are all HNK-1 stained cells NCLC and conversely are all NCLC HNK-1 positive? Second, it will be important to compare gene expression profiles of ascidian NCLC and vertebrate NC cells. This comparison may solve the important question concerning which genes or gene cascades were coopted by the vertebrate lineage during the evolution of definitive NC cells [25,72]. Moreover, some of these genes may provide additional NCLC markers to substantiate the results obtained by HNK-1 staining. Third, it must be determined whether pigment cells are the only derivatives of ascidian NCLC and conversely whether all ascidian body pigment cells are derived from NCLC. Since HNK-1 staining disappears in ascidian NCLC after the cessation of migration and differentiation, NCLC lineages and their fates in the adult can only be followed using high-resolution indelible markers. Clearly, precisely determining the fate or fates of ascidian NCLC should be a very high priority in future studies.

Studies of NCLC must also be expanded to other urochordates, cephalochordates, and non-chordate deuterostomes to obtain a more comprehensive understanding of NC ancestry.

Does amphioxus have NC cells despite its apparent lack of body pigmentation, mesenchymal cells, and placodes [28]? Whittaker [74] points out that the organs of Hess, a series of melanocytes arranged bilaterally along the length of the amphioxus neuroaxis, are candidates for NC derivatives [74]. Embryonic development of the organs of Hess should be investigated before further conclusions can be made about the status of the NC in cephalochordates. Finally, are NCLC present outside the chordates, perhaps existing in a primitive form in hemichordates or echinoderms [75], which often display intense body pigmentation?

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