Hagfish (Cyclostomata, Vertebrata): searching for the ancestral developmental plan of vertebrates

Shigeru Kuratani* and Kinya G. Ota

Summary

The phylogenetic position of the hagfish remains enigmatic. In contrast to molecular data that suggest monophyly of the cyclostomes, several morphological features imply a more ancestral state of this animal compared with the lampreys. To resolve this question requires an understanding of the embryology of the hagfish, especially of the neural crest. The early development of the hagfish has long remained a mystery. We collected a shallow-water-dwelling hagfish, *Eptatretus burgeri*, set up an aquarium tank designed to resemble its habitat, and successfully obtained several embryos. By observing the histology and expression of genes known to play fundamental roles in the neural crest, we found that the hagfish crest develops as delaminating migratory cells, as in other vertebrates. We conclude that the delaminating neural crest is a vertebrate synapomorphy that seems to have appeared from the beginning of their evolutionary history, before the splitting away of the hagfish lineage. *BioEssays* 30:167–172, 2008. © 2008 Wiley Periodicals, Inc.

Introduction

For experimental purposes, developmental biologists have established various model vertebrates such as the mouse, chicken, *Xenopus*, and zebrafish. However, phylogenetically, these all belong to the Osteichthyes, a crown group of vertebrates (Fig. 1). Ascidians and urochordates can also serve as experimental models for studying the origin of vertebrates at the cellular level, because the developmental mechanisms that establish a part of their embryonic body plan are expected to be shared with those of vertebrates, as seen in the dorsal nerve cord and the notochord. Ascidian developmental studies of the "primitive chordate" forms have contributed to our knowledge of the basic developmental program of chordates, and this type of study is now being extended into genomics.[1–4] When the recent history of evolutionary developmental ("evo-devo") studies is viewed, it is evident that information on agnathans (jawless vertebrates) is rather poor, even though these animals may show an intermediate state of developmental program linking non-vertebrate chordates and well-known vertebrate model animals (Fig. 1). Among the living vertebrates, only two groups, the hagfishes (Fig. 2) and lampreys (Fig. 3), are termed agnathans: they do not possess dorsoventrally divided movable jaws derived from the mandibular arch but instead have developed an anteroposteriorly moving tongue apparatus in the oral region.[5,6] They also lack the neck region defined by the position of paired fins and pectoral girdles.[7] Technically, the agnathans do not form a proper taxonomic group because they are paraphyletic, or a "grade" of vertebrates, by simply sharing a plesiomorphic character state: the absence of a jaw (Fig. 1). In other words, among all vertebrate species, including the fossil forms, the monophyletic crown group gnathostomes (jawed vertebrates) share and are defined by the "jaw" as a derived trait, and all other vertebrates are conventionally called agnathans. Recent molecular phylogenetics suggest that the hagfish and lampreys form a clade, the cyclostomes, which seems to have diverged from the lineage that leads to gnathostomes (Fig. 1A).[8–10] However, the relationships between hagfish, lampreys and jawed vertebrates are still controversial because the morphological traits of hagfish imply that this animal should be placed as an outgroup to the clade comprising lampreys and gnathostomes (Fig. 1B).

The idea of a monophyletic cyclostome group may be less exciting than the alternative of polyphyly for evolutionary embryologists because it implies that hagfish embryos will not tell us more than we already know from studies on the lamprey. Still, the comparison between hagfish and lamprey embryos will enable us to distinguish truly shared developmental characteristics between gnathostomes and cyclostomes from those resulting from parallel evolution between lampreys and gnathostomes. Alternatively, if the hagfish is to be placed in the outgroup of the clade comprising lampreys and gnathostomes, their embryos may reveal an intermediate stage of developmental program linking tunicates and vertebrates.[11]

Evolutionary Morphology Research Group, Center for Developmental Biology, RIKEN, Kobe, Japan.
*Correspondence to: Shigeru Kuratani, Group Director, Laboratory for Evolutionary Morphology, Center for Developmental Biology, RIKEN, 2-2-3 Minatojima-minami, Chuo, Kobe, Hyogo 650-0047, Japan.
E-mail: saizo@cdb.riken.jp
DOI 10.1002/bies.20701
Published online in Wiley InterScience (www.interscience.wiley.com).

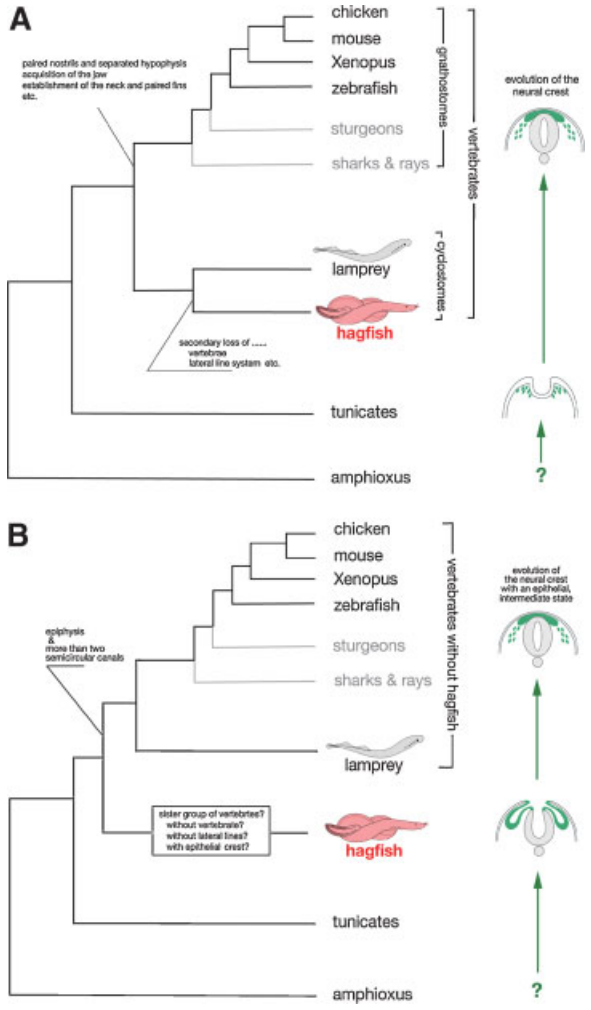

Figure 1. Two alternative views of vertebrate phylogeny lead to two different concepts of the evolution of the neural crest. **A:** Monophyly of the Cyclostomata is consistent with the actual development of the hagfish crest and with the recent observation of presumed "neural-crest-like cells" in ascidian embryos.[25] **B:** If the hagfish is regarded as a sister group of the vertebrates, Conel's description[14] of an "epithelial crest" appears to be an intermediate state of the fully evolved crest, which delaminates to produce migrating crest cells, as seen in vertebrate embryos including lamprey embryos.

A mysterious animal

The uncertainty about the phylogenetic position of the hagfish stems from the apparent loss of several structures expected in standard vertebrates, which has confused taxonomists since Linnaeus (1758).[12] For example, hagfish have lost eyes, vertebrae, and the lateral line system, although their anlagen appear during development (Fig. 1A).[13,14] Some other structures may appear truly ancestral because there is only one semicircular canal in their inner ear and no epiphysis in the brain, whereas the lamprey exhibits the epiphysis and two semicircular canals (Fig. 1B).[15] Another trait seemingly ancestral for hagfish, the neural crest, arises in the embryo as a pocket of epithelium that develops between the surface ectoderm and the neurectoderm (Fig. 1B).[16] According to Conel[16] and as reviewed by Hall,[17] this structure grows to form a nodule that differentiates directly into sensory ganglia. Kupffer (1900) also illustrated a similar histological image of the hagfish neural crest.[18] If this is true, a possible scenario for neural crest evolution is that it arose initially as an epithelial pocket, which then acquired an ability to delaminate and migrate later in evolution. Of course, this raises a question about the pluripotency of the hagfish crest cells, which appears to be restricted because these cells have a limited capability for distribution.[17]

To observe neural crest development, the embryos have to be reasonably young (midneurula to early pharyngula stages) when embryonic tissues are very delicate and easily distorted by inappropriate methods of fixation. Unfortunately, it is more than a century since Bashford Dean obtained a cluster of *Bdellostoma stoutii* eggs that contained various stages of embryos.[19] Only a few hagfish embryos have been obtained since then, and these were all at much later stages more suitable for anatomical observation of organ systems[20] than for developmental studies. Thus, it is imperative to examine the early embryonic development of the hagfish using modern methods, especially to observe the neural crest or placodes.

Obtaining hagfish embryos

Our laboratory is engaged primarily in comparative vertebrate embryology, and obtaining hagfish embryos has been our main concern. Because hagfish lack some vertebrate-defining characteristics, studying their embryos is crucial for understanding the origin of vertebrates. We have written molecular-based developmental papers on the embryo of the lamprey, another living agnathan species, but the developmental patterns of some morphological traits had to be observed directly in developing hagfish. This is because the developmental program may have been modified by the insertion of larval stages in the lamprey, or the larval stage might have been deleted secondarily in the hagfish. Around 2000, Philippe Janvier, a specialist in fossil agnathans in Paris, challenged us to obtain hagfish embryos. It is true that we Japanese scientists are gifted with a number of hagfish species in the surrounding seas because we have a fishery that deals with this animal group, and because Japanese zoology has a long tradition of cyclostome embryology, as seen in the monographs on the embryogenesis of *Lethenteron japonicum* by Saburo Hatta (Hatta S. 1891, 1897, 1900, 1901, 1907, 1915),[21–26] and on the developmental stages of *Lampetra reissneri*, a brook lamprey, by Tahara (1988).[27]

Until recently, obtaining hagfish embryos has been next to impossible for many evo-devo scientists. For example, the Royal Academy of Copenhagen offered an award to those who

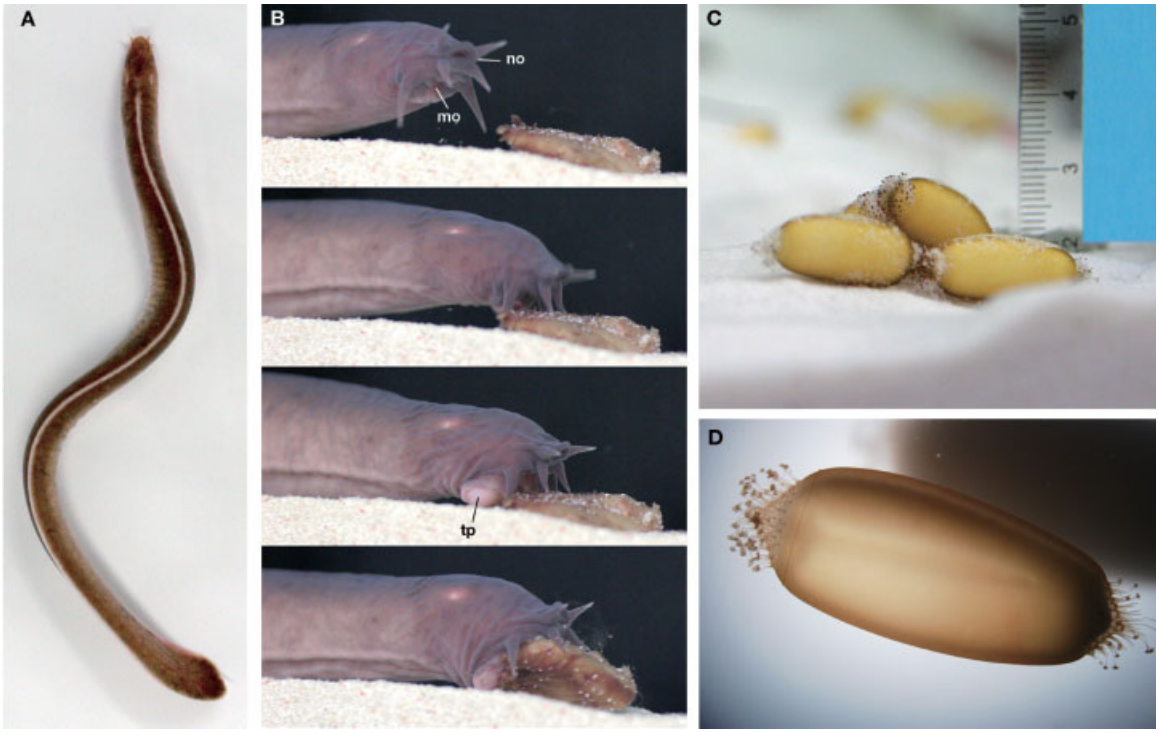

Figure 2. *Eptatretus burgeri*, a hagfish species currently being studied in our laboratory. This species is a shallow-water dweller, unlike other myxinoid species. **A:** Dorsal view of an adult. **B:** Successive video images of the moment of feeding. The fish protrudes a pair of plate-like "horny teeth" (tp) ventrally to capture the bait (cod meat). Also note the tentacle surrounding the dorsal "nostril" (no) and the ventral "mouth" (mo). **C:** Eggs laid in the laboratory aquarium. **D:** One of the eggs that contained developing embryos.

successfully collected hagfish embryos, which was, regrettably, retracted in the 1980s before anybody found one.[28] Still the eggs should be there at the bottom of the deep sea since as far as is known hagfish are born every year. Even before Kinya G. Ota, one of the authors, joined our institute, we used to meet to discuss possible strategies to tackle this problem, to talk about the animal's possible spawning season and sites, behavior, and the choice of species, and we even thought about using a remotely operated vehicle with manipulators and suction devices. Thus, in 2004, we started this project when the laboratory had expanded sufficiently to invite Ota. He had once studied hagfish genomics in Peter Holland's laboratory and knows much about Japanese fisheries.

We knew from the beginning that our efforts could fail. We had heard that many endocrinologists had tried and failed to obtain fertilized eggs and that many scientists had obtained eggs in aquarium conditions, but that these had not developed into embryos. Our goal at the time was to let the embryos develop in the eggs. We received many suggestions from various comparative endocrinologists and anatomists with hagfish experience to repeat all the preliminary experiments that did not work, including hormonal injections. It is possible that no one before had been fully committed to this task but had instead undertaken it more as a sideline of their other research, and thus they may have ignored some subtle clues. That was why we assigned Ota fully to this project, with no other commitments: he could concentrate only on obtaining embryos. We were aware that this carried the risk that Ota would either have his name as the first author of a letter in Nature or spend up to five years on this research in vain. That was a risk that we were willing to take.

We finally chose a standard method: to catch mature male and female adult hagfish, keep them in a well-conditioned tank, and wait with patience. To use this strategy, we had to collaborate with fishermen, especially to sample hagfishes in the spawning season. Indeed, Dean's success relied largely on the Chinese fisherman Ah Tack Lee, who was very familiar with the behavior of the hagfishes in Monterey Bay.

Following Dean's tracks, we made contact with a local fisherman, Osamu Kakitani, who was also very familiar with the seasonal behavior of hagfishes in the sea around Shimane Prefecture. With his help, we obtained a large number of mature male and female Japanese inshore hagfish (*Eptatretus burgeri*) living in relatively shallow water at less than 200 m. We chose this shallow-dwelling species because we intended to keep the animals in tanks under conditions as natural as

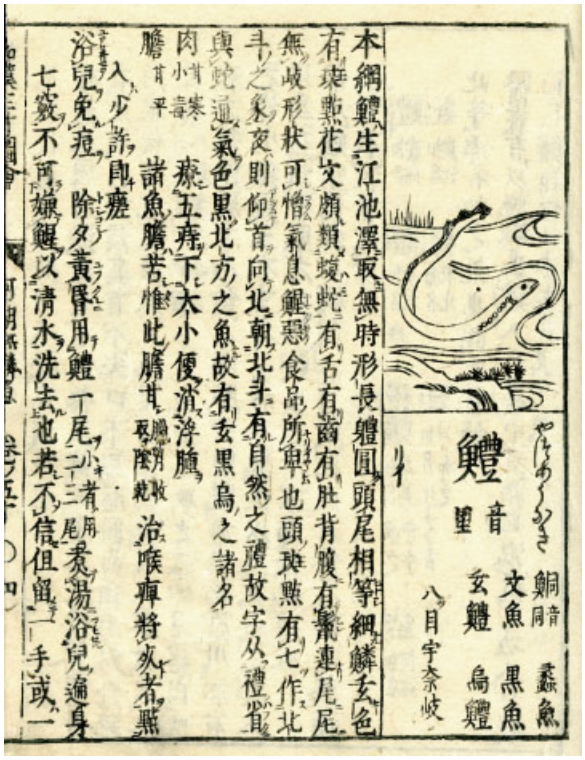

Figure 3. An old illustration of another cyclostome, the lamprey, from a Japanese encyclopedia, "Wakan-Sansai-Zue", published about 200 years ago in the Edo Era. The illustration is accurate in terms of the number of gill pores and absence of paired fins, but it was apparently recognized as one type of bony fish, and shown by the operculum and the dorsoventrally movable jaw, which are not found in the real lamprey. The hagfish does not appear in this encyclopedia.

possible. We thought it important for inducing spawning to minimize the difference in temperature and water pressure between the fish's natural habitat and the aquarium tank. The shallow-water habitat of this hagfish species was a tremendous advantage in conducting our experiments. We also kept the tank in complete darkness to mimic their habitat (see Ota and Kuratani[29] for details). As a result, in the first season of the trial, the females deposited more than 90 eggs on the bottom.

Our trick, if we could call it a trick, was to wait a little longer than other scientists had, as long as five months. The hagfish embryos that we obtained corresponded roughly to the late neurula and early pharyngula stages. It took more than four months for the embryos to become visible through the eggshell, which was much longer than estimated previously.[19] Furthermore, the developmental stages did not appear to be consistent with the time the embryos became first visible, exactly as Dean had suggested. It thus remains unclear how the fish fertilize the egg, suggesting that fertilization may have also taken place in other laboratories, but that the eggs were erroneously classed "unfertilized" and discarded before the embryos appeared.

Observations

With a large amount of yolk, the overall morphology of the hagfish embryo is reminiscent of amniote embryos rather than those of the lamprey.[11,27] The younger embryo showed—unlike the illustration of Dean's specimen—a straight neural tube whose rostral portion was differentiating into a brain primordium regionalized into several domains. Lateral to the neural tube was a pair of densely packed mesenchymal structures, or the putative trigeminal crest cells connected by a thin thread of cells (trigeminal nerve root?) with the hindbrain.[11]

Histology showed that the younger embryo, which resembled somewhat Hamburger–Hamilton's stage-10 chick embryo,[30] was at an ideal stage to observe neural crest development. Because the neural crest is expected to develop in an anterior to posterior direction, a small number of embryos will suffice to observe the entire course of crest development. The caudalmost portion (the level of presomitic mesoderm) of the embryo showed no trace of crest development.[31] However, the dorsal (or lateral) edge of the neural plate at this stage already expressed a *Sox9* cognate in the hagfish, a homeobox gene known to function in the specification of the neural crest in other vertebrates.[32–35] Rostrally, this gene expression is carried over to the delaminated and emigrating crest-like cells, which cluster in intersomitic regions. Further rostrally, similar delaminated cells were also seen under the surface ectoderm, possibly representing the pigment cell precursors. Thus, as expected, the hagfish crest-like cells seemed to proceed from the anterior to rostral direction. These somite-associated cells (which seem to correspond to ventrolateral cell populations differentiating into spinal dorsal root ganglia) and dorsolateral cell populations appeared successively, as in the typical crest cell development found in amniotes.[32,33] Moreover, topographical expression patterns of other regulatory genes such as *Pax6*, *Pax3/7* and *SoxE,* were similar to those expressed in gnathostome embryos. This suggests that the neural crest of the hagfish is specified as a part of the dorsoventral specification of the neurectoderm, based on the shared molecular mechanisms recognized in vertebrate model animals. Therefore, the delaminating neural crest and its developmental mechanisms seem to have been already established by the latest common ancestor of all vertebrates (comprising cyclostomes and gnathostomes) because the migrating cell population is known through developmental studies on model vertebrates. The observation by Conel[16] of an "epithelial crest" would have been biased by the in toto fixation of the embryos encapsulated in the eggshell. Actually, the distortion of the neuroepithelium was recapitulated by fixing one of our embryos using Dean's method.

Perspectives

The problem of the phylogenetic position of the hagfish (sister group or ingroup of vertebrates) remains unresolved. Nevertheless, we believe that our finding of delaminating neural crest cells in the hagfish drives our ideas one step toward the monophyly of cyclostomes. At least, the hypothesis of placing the hagfish basal to all the vertebrates has lost one piece of supporting evidence of the so-called "primitive neural crest arising as an epithelial pocket". We note that hagfish embryos show the vestigial lateral line system and vertebrae,[13,34] and that the loss of these traits in the adult hagfish is also likely to represent a secondary condition, not the plesiomorphic state of this animal.

A more important phylogenetic implication is that the delaminating crest is consistent with neural crest-like cells reported in tunicate larvae.[35] Arising close to the nerve cord, these cells differentiate into pigment cells by expressing many regulatory genes common to those expressed in vertebrate crest cells.[35] Although it may not be easy to show unequivocally that they represent the ancestral crest, at present it appears reasonable to assume that the delamination and subsequent migration are one of the earliest properties acquired in neural crest evolution. An intriguing question is how and in what sequence the multiple aspects of neural crest development—specification, epithelial–mesenchymal transition and differentiation—were attained successively through evolution in this lineage of cells. It will also be interesting to identify the molecular networks functioning behind these cells, if they really represent the crest cell precursor in a state of nonvertebrate chordate.[36]

To clarify the phylogenetic position of the hagfish, it will be important to characterize the developmental nature of several morphological features that appear comparatively more ancestral to lampreys and gnathostomes, such as the single semicircular canal reviewed by Janvier[15] and the apparent endodermal origin of the hypophysis.[37] For both organs, comparing the expression patterns and functions of some developmental regulatory genes will provide us with clues to understand what is unique about this animal. More importantly, histological observations are desperately scarce for this animal. In particular, information on early embryos from the late neurula to pharyngula stages is missing, yet this is fundamentally important to understanding the peculiar embryonic pattern of the pharynx in this animal. Now, at last, hagfish embryos have turned out to be obtainable and provide a promising model for comparison. There is a lot of work ahead of us.

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