doi:10.1038/nature05633

LETTERS

Hagfish embryology with reference to the evolution of the neural crest

Kinya G. Ota[1], Shigehiro Kuraku[1] & Shigeru Kuratani[1]

Hagfish, which lack both jaws and vertebrae, have long been the subject of intense interest owing to their position at a crucial point in the evolutionary transition to a truly vertebrate body plan[1–4]. However, unlike the comparatively well characterized vertebrate agnathan lamprey, little is known about hagfish development. The inability to analyse hagfish at early embryonic stages has frustrated attempts to resolve questions with important phylogenetic implications, including fundamental ones relating to the emergence of the neural crest[1,5,6]. Here we report the obtainment of multiple pharyngula-stage embryos of the hagfish species *Eptatretus burgeri* and our preliminary analyses of their early development. We present histological evidence of putative neural crest cells, which appear as delaminated cells that migrate along pathways corresponding to neural crest cells in fish and amphibians[2,7–11]. Molecular cloning studies further revealed the expression of several regulatory genes, including cognates of *Pax6*, *Pax3/7*, *SoxEa* and *Sox9*, suggesting that the hagfish neural crest is specified by molecular mechanisms that are general to vertebrates. We propose that the neural crest emerged as a population of de-epithelialized migratory cells in a common vertebrate ancestor, and suggest that the possibility of classical and molecular embryology in hagfish opens up new approaches to clarifying the evolutionary history of vertebrates.

Tracing the evolutionary origin of vertebrates requires careful comparative studies of this group and its closest phylogenetic relatives. The embryonic development of non-vertebrate chordates has

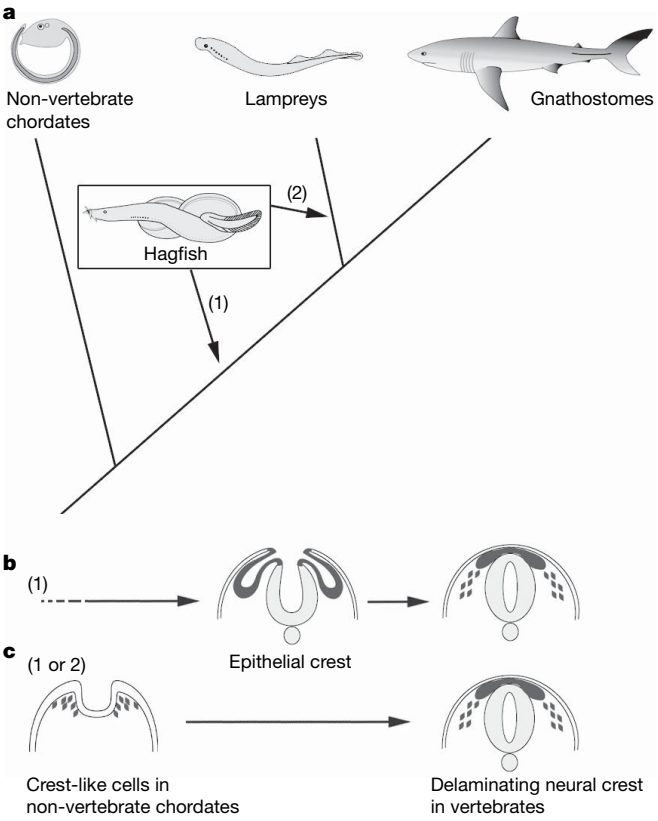

Figure 1 | Evolution of vertebrates and neural crest. **a**, The hagfish may be either the sister group of all the other vertebrates (1), or that of lampreys (2). **b**, **c**, Hypotheses on neural crest evolution. **b**, The former hypothesis (1) would agree with the scenario that neural crest evolution had an intermediate epithelial state. **c**, Alternatively, the crest might have already been established as a population of delaminating cells in the common ancestor to all vertebrates, and this would be coherent with both hypotheses (1 or 2).

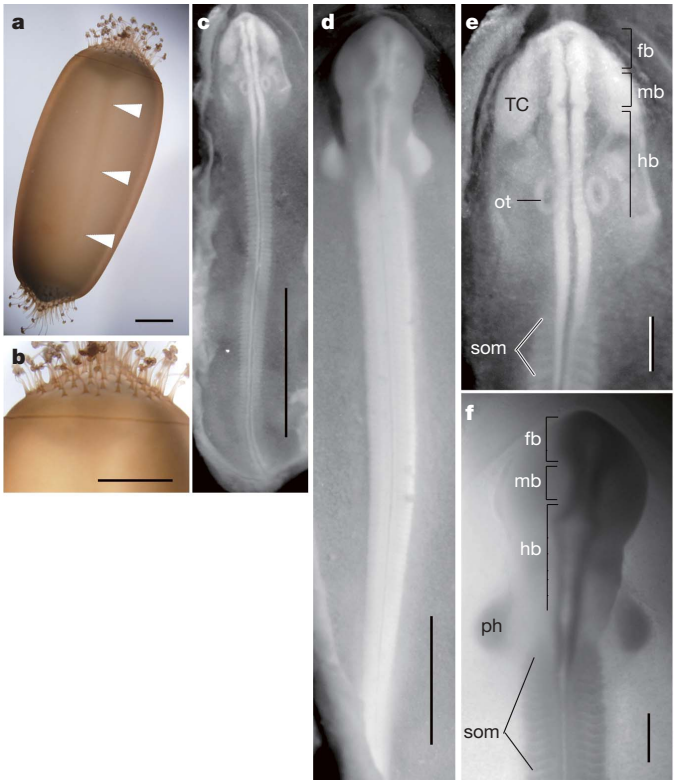

Figure 2 | Embryos of *Eptatretus burgeri*. **a**, **b**, An egg with a 14.3-mm pharyngula-stage embryo inside (arrows). **b**, Higher magnification of the head. **c**, A 7.4-mm embryo, corresponding to the late neurula stage. **d**, A 14.3-mm embryo, corresponding to the early pharyngula stage. **e**, The head of the 7.4-mm embryo. **f**, The head of the 14.3-mm embryo. fb, forebrain; hb, hindbrain; mb, midbrain; ot, otic pit; ph, pharyngeal wall; som, somites; TC, trigeminal crest cells. Scale bars, 5 mm (**a**–**d**); 1 mm (**e**, **f**).

[1]Laboratory for Evolutionary Morphology, Center for Developmental Biology, RIKEN, Kobe 650-0047, Japan.

been extensively studied, as has that of the agnathan (jawless vertebrates) lampreys; however, the development of hagfish, another agnathan group, remains poorly understood, largely owing to the lack of access to embryos[1,2,4,12]. It has been more than one-hundred years since the first series of *Bdellostoma stouti* embryos was obtained[13], and limited attempts have been made since, yielding only a few embryos at later stages in development[4]. The phylogenetic relationship between hagfish, lampreys and gnathostomes (jawed vertebrates) is unresolved, but the hagfish, which lack vertebrae, are often located by morphologists and physiologists as a sister group to the vertebrates (Fig. 1a)[1,2,6]. If this phylogenetic assignment is correct, hagfish embryos might presumably exhibit primitive features not found in lampreys, meaning that the study of this taxon might provide new insights into the evolutionary pathways that led to the emergence of vertebrates.

The ability to make histological observations of hagfish embryos opens up particularly intriguing possibilities for developing a better understanding of the origins of two vertebrate-specific innovations: the ectodermal placode and the neural crest[14]. The presence of placodes has already been suggested in the early hagfish pharyngula[3,15]. As for the crest, it has been suggested that in hagfish this develops as an epithelial pocket arising between the surface ectoderm and neurectoderm, and not as a population of delaminated migrating cells, as is the case in vertebrate embryos (Fig. 1b)[16]. If this model is correct, the delaminated crest would be specific to lampreys and gnathostomes, and absent in hagfish. Even if, as suggested by recent molecular data[17–19], lampreys and hagfish do form a monophyletic group (cyclostomes), analyses of hagfish embryos will still help to define more clearly gnathostome-specific features not shared by cyclostomes. Thus, whichever model is correct, the ability to study hagfish embryos is an absolute prerequisite to developing a better understanding of their true phylogenetic position.

We chose to study a shallow-water hagfish species, *Eptatretus burgeri*, because, in contrast to more deep-water-dwelling species, we felt

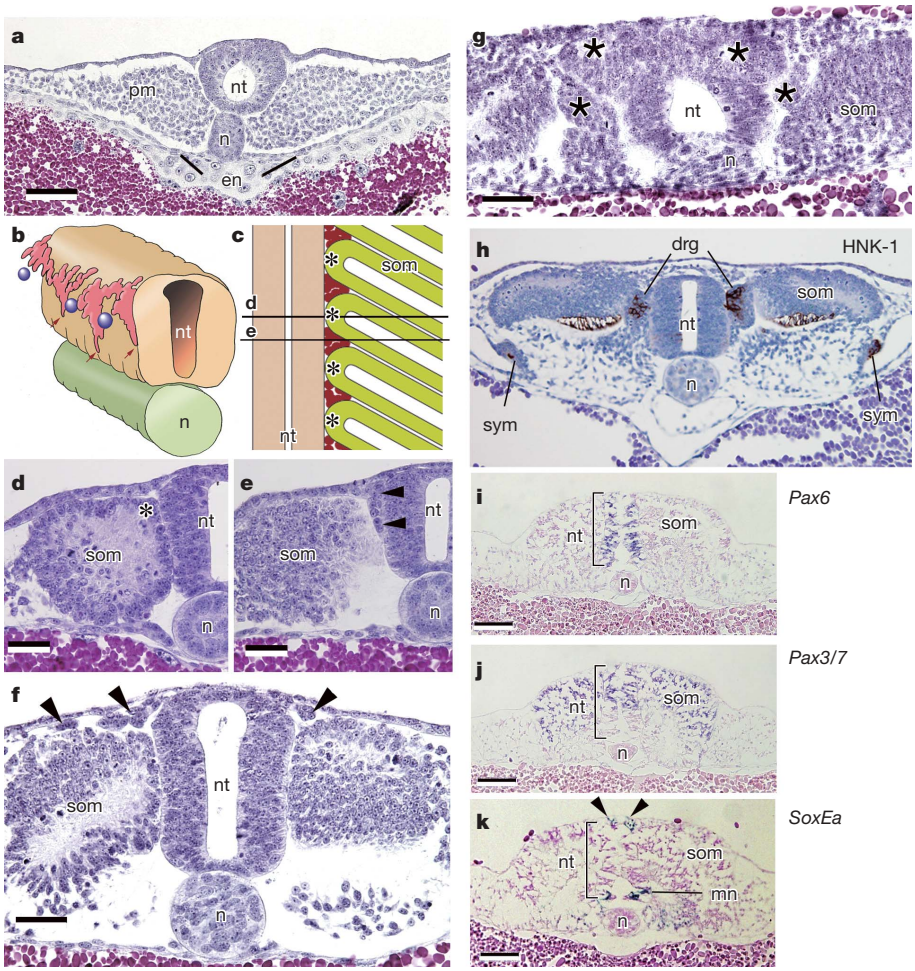

Figure 3 | Neural crest cells in *E. burgeri*. **a**–**e**, Putative neural crest cells in hagfish embryos. **a**–**f**, A 7.4-mm embryo. **a**, No neural crest cells are found in the caudal-most region of the trunk. **b**, Illustration of neural crest cell distribution in the same embryo on the basis of three-dimensional reconstruction from the histological sections; seen from the anterior oblique view. Red arrows indicate the ventrally migrating crest cells occurring at the same intervals as the apices of somites shown by purple circles. **c**, Schematic representation of a horizontal section of the embryo to show the planes of sections **d** and **e**. **d**, A transverse section at the mid-somite level. No putative crest cells are found between the somite (som) and the neural tube (nt). Asterisks in **c** and **d** indicate the apex of each somite. **e**, A transverse section at the intersomitic level. Putative crest cells (arrowheads) are filling in a space between the somite and the neural tube. **f**, Slightly rostral to **a**, showing putative crest cells (arrowheads) beneath the ectoderm. **g**, Transverse section of a 13.4-mm embryo fixed *in toto*. Neuroepithelial pockets (asterisks) appear next to the neural tube. **h**, A 14.3-mm embryo immunostained with HNK-1 antibody. Differentiated neurons including dorsal root ganglia (drg) and sympathetic ganglia (sym), as well as a medial cell population of somites, are stained. **i**–**k**, Expression of the indicated hagfish cognates of the genes involved in vertebrate neural crest specification in the trunk level of a 13.1-mm embryo. *Pax6* is expressed in the entire neural tube (**i**); *Pax3/7* in its dorsal part (**j**). *Pax3/7* is also expressed in the dorsal somites as in gnathostome embryos. **k**, *SoxEa* is expressed in the putative motor neurons (mn) and the pre-migratory neural crest (arrowheads), but not in the migrating crest cells. Brackets indicate the dorsoventral extent of the neural tube. drg, dorsal root ganglia; en, endoderm; mn, motor neurons; n, notochord; nt, neural tube; pm, paraxial mesoderm; som, somite; sym, sympathetic ganglia. Scale bars, 100 μm.

it would be easier for us to recreate their natural habitat in the aquarium[4]. We prepared an aquarium tank in which a large number of males and brood females were kept at low temperature, in an attempt to reproduce the hagfish spawning environment[4]. The animals deposited a total of ninety-two eggs, among which we found seven developing embryos visible through the eggshells in the period from five to seven months after deposition (Fig. 2a, b). These embryos were at four different developmental stages (Supplementary Table 1), confirming the finding that hagfish embryogenesis is asynchronous[13]. It is worth noting that *E. burgeri* takes much longer to hatch than a previous estimation[13] of two months for *B. stouti*, leading us to suspect that some of the eggs previously obtained by other laboratories may have been discarded before development had begun.

To observe the embryos in greater detail, we tested different methods of fixation and compared their histological and whole-mount appearance. As found in the study of ref. 13, which fixed the embryos *in toto* (encapsulated in shells), removal of the eggshell seemed necessary to avoid distortion of specimens. Distortion was enormous in the neural tube of the specimens of ref. 13 (Supplementary Fig. 1), probably owing to unequal swelling and shrinkage of various tissues in a confined space. By fixing an embryo *in toto*, we were able to reproduce the neural tube distortion, and found epithelial pockets lateral to the neural tube, such as were described previously[16] on examination of the specimen of ref. 13 (Fig. 3g; see also Supplementary Fig. 1a).

When we fixed 14.3-mm and 7.4-mm embryos by removing the eggshells, we found well organized brain primordia reminiscent of those in other vertebrate embryos (Fig. 2c–f). On sectioning, we did not find any epithelial pockets, but observed putative migrating neural crest cells at the trunk level (Fig. 3e, f). The crest cells appeared to have delaminated in close proximity to the dorsal neural tube, by a process that seemed to proceed in an anterior to posterior direction (Fig. 3a, e, f), and populated in a segmental pattern associated with somites, as is typical of vertebrate crest cells (Fig. 3b–e)[2,11]. Unlike in amniote embryos[2,11], none of the crest cells migrated into the somites. We performed immunostaining with the monoclonal antibody HNK-1, which is known to recognize migrating crest cells in some vertebrates[11]; however, although this monoclonal antibody did not detect any crest cells, it did label the differentiated dorsal root ganglia that developed in an older embryo (Fig. 3h). These ganglia are also located intersomitically[20], as the crest cells are at previous stages.

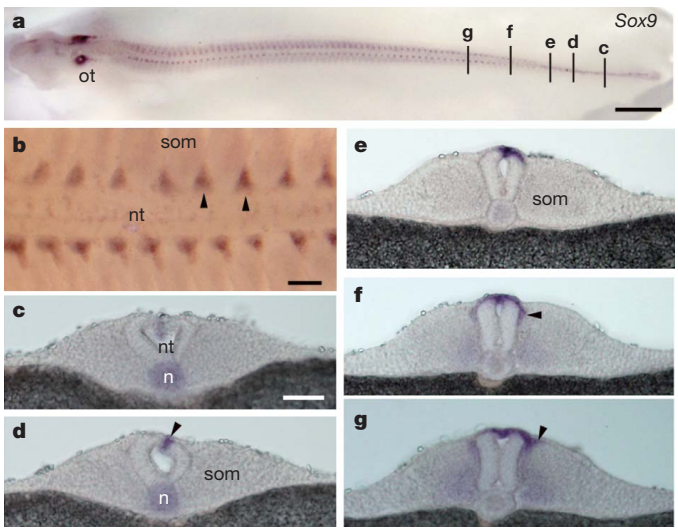

Figure 4 | *Sox9* expression in the neural crest of *E. burgeri*. **a**, **b**, A whole-mount embryo hybridized with a *Sox9* probe. *Sox9* is expressed in otocyst (ot) and neural crest cells populating between somites (som), as in gnathostome embryos. Lines indicate the levels of sections shown in **c**–**g**. **b**, Enlargement of the trunk level. *Sox9*-positive neural crest cells (arrowheads) are predominantly located between intersomitic spaces. **c**–**g**, Transverse sections of the same embryo, shown from caudal to rostral levels. *Sox9* is expressed in the notochord (n) and the dorsal neural tube (nt), representing the pre-migratory neural crest at the caudal-most level (**c** and **d**), and also in the migrating crest cells (arrowheads) at more rostral levels (**e**–**g**). Scale bars, 1 mm (**a**); 100 μm (**b**–**g**).

To investigate the hagfish neural crest at the molecular level, we isolated two *Pax* genes (*Pax6* and *Pax3/7*) as markers for regionalization of neuroepithelium[21,22], and a *Snail* gene homologue (*SnailA*) and two *SoxE* genes (*SoxEa* and *Sox9*) as candidate neural crest markers[23–25] (Supplementary Figs 2–5). We conducted *in situ* hybridization and found that the expression patterns of *Pax6* and *Pax3/7* were identical to those in the gnathostome neural tube (Fig. 3i, j). Although *SnailA* was not expressed in the putative crest (Supplementary Fig. 6), both the *SoxE* genes (*SoxEa* and *Sox9*) were detected strongly in the neural crest at the trunk level (Figs 3k and 4). In the whole-mount embryo, *Sox9* was also expressed in the segmentally arranged crest cells (Fig. 4), corresponding to the distribution patterns of the putative crest cells in the histological sections (Fig. 3b–e).

These findings indicate that the genetic programmes that specify both the neural crest cells and the overall embryonic architecture of the hagfish resemble those of the typical vertebrate scheme. We have also not detected any feature that would be present in other chordates but not in vertebrates. The absence of *Snail* transcripts in the hagfish neural crest may indicate either an evolutionary change in gene regulation[26] or the presence of another *Slug*-like paralogue in the hagfish; further phylogenetic studies will be needed to resolve this question. Given this new histological and genetic evidence, we suggest that the neural crest probably existed as a population of delaminating and migrating cells in the common ancestor of the entire vertebrate clade, and thus that its origin should be sought in non-vertebrate chordates (Fig. 1a, c). Notably, this model is consistent with the recent finding of 'putative crest cells' in tunicate embryos[27]. These cells not only express a similar set of genes compared to those characteristic of vertebrate neural crest cells, but they also migrate.

Our data indicate that these embryological features, which have been considered to be specific to vertebrates, were in fact already present in the common ancestor of hagfish, lampreys and gnathostomes, and that, given the time of the divergence between hagfish and other vertebrates, their origin could date back to 500 million years ago (Cambrian period)[28,29]. In addition to the origin of neural crest, a number of other questions surrounding hagfish embryogenesis remain, including the origin and development of ectodermal placodes, thyroid and adenohypophysis, the configuration of the cephalic endoderm, and the origin of direct development in their life cycle[4,12,13,30]. Although the present study of hagfish development does not provide conclusive evidence towards resolving the phylogenetic position of this taxon (Fig. 1a), this organism nonetheless remains a key model for exploring vertebrate evolution, and, as we have shown, is now available for use in molecular embryological approaches to address fundamental questions in evolutionary developmental biology.

METHODS

Sample collection and aquarium maintenance. Adult males and females of *E. burgeri* were collected using eel traps from a depth of 25–100 m in the Japan Sea off Shimane prefecture from September to October 2005. After the hagfish were transferred to a laboratory aquarium (at 16 °C) and sexed by manipulation, 25 individuals (13 males and 12 females) were maintained in the aquarium tank lined with some potentially favourable substrates, including fine-grain sands and oyster shells (1,000 l, 16–17 °C), from 10 October 2005 to 30 April 2006. Haematoxylin and eosin staining and HNK-1 immunostaining were performed by standard protocols. Fragments of *Pax6*, *Pax3/7*, *SoxEa*, *Sox9* and *SnailA* were amplified by degenerate polymerase chain reaction with reverse transcription and isolated by the TOPO TA cloning kit dual promoter (Invitrogen). *In situ* hybridization was carried out in a Ventana automated machine (Ventana Medical Systems). Detailed protocols are described in Supplementary Information.

Received 5 October 2006; accepted 22 January 2007.
Published online 18 March 2007.

1. Janvier, P. *Early Vertebrates* (Oxford Univ. Press, Oxford, 1996).
2. Hall, B. K. *The Neural Crest in Development and Evolution* (Springer, New York, 1999).
3. Wicht, H. & Northcutt, R. G. Ontogeny of the head of the Pacific hagfish (*Eptatretus stouti*, Myxinoidea): development of the lateral line system. *Phil. Trans. R. Soc. Lond. B* **349,** 119–134 (1995).
4. Ota, K. G. & Kuratani, S. The history of scientific endeavors towards understanding hagfish embryology. *Zoolog. Sci.* **23,** 403–418 (2006).
5. Holmgren, N. On two embryos of *Myxine glutinosa*. *Acta Zoologica* **27,** 1–90 (1946).
6. Løvtrup, S. *The Phylogeny of Vertebrata* (Wiley, New York, 1977).
7. Bronner-Fraser, M. Environmental influences on neural crest cell migration. *J. Neurobiol.* **24,** 233–247 (1993).
8. Collazo, A., Bronner-Fraser, M. & Fraser, S. E. Vital dye labelling of *Xenopus laevis* trunk neural crest reveals multipotency and novel pathways of migration. *Development* **118,** 363–376 (1993).
9. Eisen, J. S. & Weston, J. A. Development of the neural crest in the zebrafish. *Dev. Biol.* **159,** 50–59 (1993).
10. McCauley, D. W. & Bronner-Fraser, M. Neural crest contributions to the lamprey head. *Development* **130,** 2317–2327 (2003).
11. Le Douarin, N. & Kalcheim, C. *The Neural Crest* (Cambridge Univ. Press, Cambridge, 1999).
12. Gorbman, A. Hagfish development. *Zoolog. Sci.* **14,** 375–390 (1997).
13. Dean, B. On the embryology of *Bdellostoma stouti*. A general account of myxinoid development from the egg and segmentation to hatching. In *Festschrift zum 70ten Geburststag Carl von Kupffer* 220–276 (Gustav Fischer, Jena, 1899).
14. Gans, C. & Northcutt, R. G. Neural crest and the origin of vertebrates: A new head. *Science* **220,** 268–274 (1983).
15. Wicht, H. & Tusch, U. in *The Biology of Hagfish* (eds Jørgensen, J. M., Lomholt, J. R., Weber, R. E. & Malte, H.) 431–451 (Chapman & Hall, London, 1998).
16. Conel, J. L. The origin of the neural crest. *J. Comp. Neurol.* **76,** 191–215 (1942).
17. Mallatt, J. & Sullivan, J. 28S and 18S rDNA sequences support the monophyly of lampreys and hagfishes. *Mol. Biol. Evol.* **15,** 1706–1718 (1998).
18. Takezaki, N., Figueroa, F., Zaleska-Rutczynska, Z. & Klein, J. Molecular phylogeny of early vertebrates: monophyly of the agnathans as revealed by sequences of 35 genes. *Mol. Biol. Evol.* **20,** 287–292 (2003).
19. Furlong, R. F. & Holland, P. W. Bayesian phylogenetic analysis supports monophyly of ambulacraria and of cyclostomes. *Zoolog. Sci.* **19,** 593–599 (2002).
20. Goodrich, E. S. *Studies on the Structure and Development of Vertebrates* (McMillan, London, 1930).
21. Callaerts, P., Halder, G. & Gehring, W. J. *PAX-6* in development and evolution. *Annu. Rev. Neurosci.* **20,** 483–532 (1997).
22. Goulding, M. D., Chalepakis, G., Deutsch, U., Erselius, J. R. & Gruss, P. Pax-3, a novel murine DNA binding protein expressed during early neurogenesis. *EMBO J.* **10,** 1135–1147 (1991).
23. Cheung, M. & Briscoe, J. Neural crest development is regulated by the transcription factor Sox9. *Development* **130,** 5681–5693 (2003).
24. McCauley, D. W. & Bronner-Fraser, M. Importance of SoxE in neural crest development and the evolution of the pharynx. *Nature* **441,** 750–752 (2006).
25. Nieto, M. A., Sargent, M. G., Wilkinson, D. G. & Cooke, J. Control of cell behavior during vertebrate development by Slug, a zinc finger gene. *Science* **264,** 835–839 (1994).
26. Locascio, A., Manzanares, M., Blanco, M. J. & Nieto, M. A. Modularity and reshuffling of *Snail* and *Slug* expression during vertebrate evolution. *Proc. Natl Acad. Sci. USA* **99,** 16841–16846 (2002).
27. Jeffery, W. R., Strickler, A. G. & Yamamoto, Y. Migratory neural crest-like cells form body pigmentation in a urochordate embryo. *Nature* **431,** 696–699 (2004).
28. Shu, D. G. *et al.* Head and backbone of the Early Cambrian vertebrate *Haikouichthys*. *Nature* **421,** 526–529 (2003).
29. Kumar, S. & Hedges, S. B. A molecular timescale for vertebrate evolution. *Nature* **392,** 917–920 (1998).
30. Gorbman, A. & Tamarin, A. Early development of oral, olfactory and adenohypophyseal structures of agnathans and its evolutionary implications. In *Evolutionary Biology of Primitive Fishes* (eds Foreman, R. E., Gorbman, A., Dodd, J. M. and Olsson, R.) 165–185 (Plenum, New York, 1985).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank O. Kakitani for the sample collection, H. Nagashima and Y. K. Ohya for technical advice, and R. Ladher and D. Sipp for critical reading of this manuscript. This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Author Contributions K.G.O. performed sample collection, maintenance of the aquarium tank, molecular cloning and *in situ* hybridization. S. Kuraku was particularly engaged in isolation of *Snail* family genes and performed phylogenetic analyses. S. Kuratani operated on the hagfish embryos and conducted histological analysis. K.G.O. and S. Kuratani wrote the manuscript. All of the authors discussed the results and commented on the manuscript.

Author Information Sequences for *Pax6*, *Pax3/7*, *Snail*, *SoxEa* and *Sox9* from *E. burgeri* are deposited in DNA Data Bank of Japan (DDBJ) under accession numbers AB270704, AB270703, AB288229, AB288230 and AB270702, respectively. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to S. Kuratani (saizo@cdb.riken.jp).