JOURNAL OF EXPERIMENTAL ZOOLOGY (MOL DEV EVOL) 302B:458–468 (2004)

Developmental Fate of the Mandibular Mesoderm in the Lamprey, *Lethenteron japonicum:* Comparative Morphology and Development of the Gnathostome Jaw With Special Reference to the Nature of the Trabecula Cranii

SHIGERU KURATANI*[1], YASUNORI MURAKAMI†[1], YOSHIAKI NOBUSADA[2], RIE KUSAKABE[1], AND SHIGEKI HIRANO[3]
[1]*Laboratory for Evolutionary Morphology, Center for Developmental Biology, RIKEN, Kobe, Hyogo 650-0047, Japan*
[2]*Department of Biology, Okayama University, Okayama 700-8530, Japan*
[3]*Department of Medical Technology, School of Health Sciences, Faculty of Medicine, Niigata University, 2-746, Asahimachi, Niigata 951-8518, Japan*

ABSTRACT The vertebrate jaw is a mandibular-arch derivative, and is regarded as the synapomorphy that defines the gnathostomes. Previous studies (Kuratani et al., Phil. Trans. Roy. Soc. 356:15, 2001; Shigetani et al., Science 296:1319, 2002) have suggested that the oral apparatus of the lamprey is derived from both the mandibular and premandibular regions, and that the jaw has arisen as a secondary narrowing of the oral patterning mechanism into the mandibular-arch domain. The heterotopy theory of jaw evolution states that the lamprey upper lip is a premandibular element, leaving further questions unanswered as to the homology of the trabecula in the lamprey and gnathostomes, and to the morphological nature of the muscles in the upper lip. Using focal injection of vital dyes into the cheek process core of lamprey embryos, we found that the upper lip muscle and trabecula are both derived from mandibular mesoderm. Secondary movement of the muscle primordium is also evident when the expression of the early muscle marker gene, *LjMA2,* is visualized. A nerve-fiber labeling study revealed that the upper lip muscle-innervating neurons are located in the rostral part of the brain stem, where the trigeminal motor nuclei are not found in gnathostomes. We conclude that the lamprey upper lip is composed of premandibular ectomesenchyme and a lamprey-specific muscle component derived from the mandibular mesoderm innervated by lamprey-specific motoneurons. Furthermore, the lamprey trabecula is most likely equivalent to a mesodermally derived neurocranial element, similar to the parachordal element in gnathostomes, rather than to the neural-crest-derived prechordal element. *J. Exp. Zool. (Mol. Dev. Evol.) 302B: 458–468, 2004.*

The origin of the vertebrate jaw, which is the synapomorphy that defines the gnathostomes, has been and remains an intriguing issue of vertebrate evolution. To address this question, the lamprey, a living jawless vertebrate, can serve as a model for developmental and embryological comparisons with which to determine the event that gave rise to this evolutionary novelty (reviewed by Mallatt, 1996; and by Kuratani et al., 2001).

The biting jaw of the gnathostomes is a derivative of the rostralmost pharyngeal arch, the mandibular arch (Fig. 1A). Therefore, the evolution of the jaw is thought to have occurred through the transformation of the rostralmost branchial arch (Gegenbaur, 1898; Goodrich, '30; Gregory, '33; de Beer, '31, '37; Jarvik, '80; Janvier, '96; reviewed by Kuratani et al., 2001). From this classical concept, several different lines of thought have developed. One theory suggests that the velum, a pumping apparatus arising in the embryonic oropharyngeal membrane (Kuratani

†Yasunori Murakami's present address: Institut de Genetique et de Biologie Moleculaire et Cellulaire (IGBMC), CNRS/INSERM/ULP, CU de Strasbourg, BP 10142-67404 Illkirch Cedex, France
*Correspondence to: Shigeru Kuratani, 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
Received 31 March 2004; Accepted 3 May 2004
Published online 12 August 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jez.b.21011

et al., 2001), was articulated dorsoventrally and differentiated into the jaw (reviewed by Carroll, '93; Janvier, '96). However, there is no evidence to clarify whether or not the gnathostome jaw arose from an ancestral differentiated velum, and the embryonic structure equivalent to the velum does not seem to persist into the adult gnathostome (Kuratani et al., 2001). Other authors deny the velar origin of the jaw, and assert that the upper and lower jaw elements are already found in agnathan vertebrates as a reduction of the premandibular mouth and simultaneous enlargement of the mandibular arch to facilitate respiration, which led to the establishment of the gnathostome jaw (Mallatt, '96).

In a series of previous studies, we proposed a hypothesis referred to as the heterotopic theory of the vertebrate jaw (Fig. 1; see Kuratani et al., '99, 2001; Shigetani et al., 2002). By examining the embryonic head of the lamprey, we found that the upper lip ectomesenchyme (see Horigome et al., '99; Kuratani et al., '99, 2001), which surrounds the definitive cephalic mesodermal element (premandibular mesoderm), is located rostral to the mandibular arch, unlike the upper jaw of gnathostome embryos (Fig. 1B; Kuratani et al., 2001). The ectomesenchyme that participates in upper lip formation is thus of premandibular origin and is not homologous to any mandibular derivatives, but is quite closely related to the tissue that differentiates into the prechordal region of the neurocranium (Fig. 1A; ''prechordal cranium''; Couly et al., '93) of the gnathostomes. Here, ''premandibular'' denotes the region rostral to the mandibular arch, not necessarily assuming the presence of a premandibular ''arch''; see Kuratani et al., 2001 and Shigetani et al., 2000, 2002 for premandibular ectomesenchyme. Therefore, topographical reorganization of the cephalic ectomesenchyme, or reassignment of the conserved molecular cascades involved in epigenetic interactions to different subsets of the ectomesenchyme, were assumed to have occurred in the transition from the agnathan to gnathostome stages of evolution (Fig. 1B; Shigetani et al., 2002; see also Kuratani, 2003). This argument denies the morphological homologies of the upper and lower lips of the lamprey with the upper and lower jaws of the gnathostomes, respectively.

The heterotopic theory reconciles the expression of homologous regulatory genes in nonhomologous tissues by heterotopy—changes in developmental sites during evolution (Haeckel, 1875; reviewed by Hall, 1998). Heterotopy implies the disruption of ancestral developmental constraints, leading to the loss of morphological homologies, as is actually seen in the transition from agnathans to gnathostomes. Such a scenario fits the definition of evolutionary novelty (Wagner and Müller, 2002).

The model of jaw evolution illustrated above raises some questions of comparative morphology, which are still unresolved. Firstly, the muscles in the upper lip are innervated by a branch of the maxillomandibular nerve (Johnston, '05; Song and Boord, '93), which is inconsistent with the morphological concept of the ''mandibular arch''. Where these muscles originate is unknown. The premandibular mesoderm of the lamprey, which is primarily located in the upper lip primordium (Fig. 1B), has been described as differentiating into the extrinsic eye muscle (Koltzoff, 1901), as in the gnathostomes. Although this idea is purely speculative, it is supported by the expression pattern of the *Pitx* homolog in the lamprey, as reported recently (Boorman and Shimeld, 2002). Secondly, if the origin of the upper lip ectomesenchyme is close to the gnathostome trabecular cartilage, what is the morphological identity of the cartilage of the same name in the lamprey? Alternatively, what is the developmental origin of the lamprey trabecula? The morphological identity and evolutionary origin of the trabecula have long been debated, together with the question of the ''premandibular arch'' in vertebrates (Johnels, '48; reviewed by Gregory, '33; de Beer, '31, '37; Janvier, '96; and by Kuratani et al., '97a, 2001; Fig. 1A). Experimental studies are necessary to determine the morphological patterning of the rostral head mesenchyme in the lamprey, to provide clues to the solutions of these questions.

The aim of the present study was to provide some experimental data and further observations and discussion on the developmental fate and behavior of the mandibular mesoderm of the lamprey, *Lethenteron japonicum*. By applying vital dyes and observing the gene expression that defines subsets of developing muscles, we have collected supplementary data to support the heterotopic theory of vertebrate jaw evolution. This study also aimed to explain the morphological nature and the evolutionary origin of the lamprey trabecula.

MATERIALS AND METHODS

Embryos

Mature male and female lampreys, *Lethenteron japonicum,* were collected in a tributary of the

Miomote River, Niigata, or purchased from a local fishery of Hokkaido, Japan, during the breeding seasons (early June) of 2001 through to 2003. The eggs were artificially fertilized and kept in 10% Steinberg solution (Steinberg, '57) at 16°C. Embryonic stages were assessed morphologically according to the sequence established by Tahara ('88) for *L. reissneri,* a brook lamprey species closely related to *L. japonicum.*

Focal injections of DiI and DiO

Based on the method described by Shigetani et al. (2000), focal injections of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) and 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO) were made into the mandibular mesoderm. Stage 21 or younger lamprey embryos (Tahara, '88), in which the premandibular mesoderm has not yet invaded the cheek process and the process contains only the mandibular mesoderm, were used. Using a fine glass pipette, a solution of dyes was injected into the left cheek process using a Pneumatic PicoPump (PV830, World Precision Instruments, Inc., Sarasota, FL USA). Embryos were then incubated for a further 2-7 days and were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 2 h at room temperature (RT). They were rinsed briefly with 0.9% NaCl and immersed in 30% glycerol in distilled water. The embryos were mounted on a depression slide glass and observed with a fluorescence microscope.

Retrograde labeling of neurons

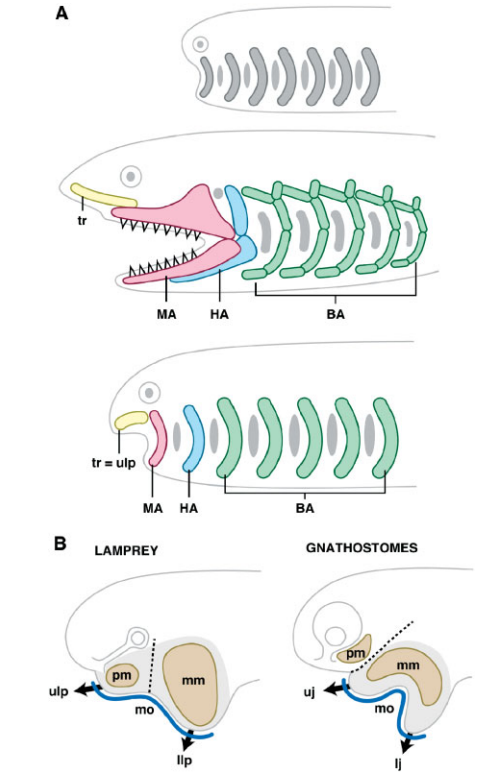
Rhodamine- or fluorescein-conjugated dextrans (Sigma, St Louis, MO, USA) were injected into the spinal cord or pharyngeal arches of the embryo to label the reticulospinal or branchial motor neurons, respectively, according to the method described by Glover ('95). The injected embryos were incubated at RT for 30 min to allow the dextran to label neurons retrogradely. Embryos were then washed with 10% Steinberg solution, and fixed in 4% PFA in PBS. The fixed specimens were dehydrated, and clarified with a 1:2 mixture of benzyl alcohol and benzyl benzoate. Labeled neurons were then examined using a fluorescence microscope.

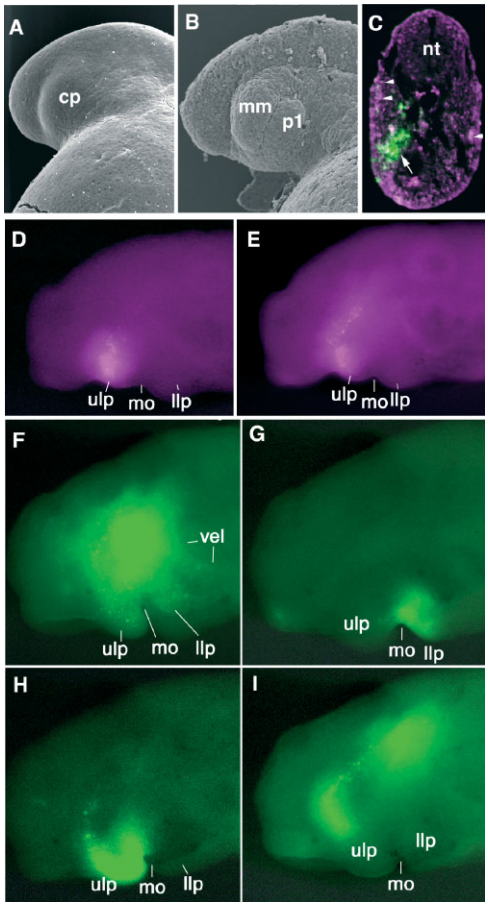
Whole-mount in situ hybridization

Whole-mount in situ hybridizations were performed as previously described (Murakami et al., 2001). Fixed embryos were dehydrated and stored in

Fig. 1. Origin of the gnathostome jaw. A. Top: generally assumed prototype of the vertebrate head with undifferentiated array of pharyngeal arch skeleton. Middle: primitive gnathostome similar to extant shark possessing the mandibular arch (MA; pink) differentiated as a biting jaw, with the hyoid arch (HA; blue) as the jaw suspension, and the real branchial arches (BA; green) that are basically of the same morphological pattern. Note that both the upper and lower jaws are derived from a single arch (MA).There is a pair of rod-like cartilages called the trabeculae (tr) in front of the mandibular arch as the anlage of the neural-crest-derived prechordal neurocranium. This cartilage used to be regarded as a premandibular arch. Bottom: schematic representation of the pre-gnathostome ancestor in terms of the heterotopy theory (Shigetani et al., 2002), showing a morphology similar to that of the ammocoete larva of the lamprey. Three types of morphological identities can be discerned, as in the gnathostomes (see Kuratani et al., 2001). In this animal, the oral apparatus is formed of both the premandibular and mandibular ectomesenchyme; the upper lip (ulp) of this animal is derived from the ectomesenchyme equivalent that gives rise to the trabecula in the gnathostomes. B. Heterotopy theory of the origin of the vertebrate jaw. In the lamprey embryo, the premandibular ectomesenchyme surrounding the premandibular mesoderm (pm) differentiates into the upper lip dorsal to the mouth (mo), and the mandibular ectomesenchyme around the mandibular mesoderm (mm) into the lower lip (llp). These portions of the ectomesenchyme commonly express *Dlx1* cognate (colored gray) to pattern the oral apparatus through epigenetic interaction with ectodermally derived growth factors (blue line). In gnathostome development, a similar interaction occurs only in the domain of the mandibular arch, and the premandibular ectomesenchyme does not express the *Dlx1* cognate. Dotted line indicates the morphological boundary between premandibular and mandibular ectomesenchyme (see Shigetani et al., 2000, 2002 for details). u*j,* upper jaw; lj, lower jaw.

Fig. 2. Dye injections into the lamprey mandibular mesoderm I. A. Scanning electron micrograph of a stage 21 lamprey embryo, left lateral view. The cheek process (cp) protrudes on the lateral aspect of the head. B. The surface ectoderm of the same stage embryo was removed to show the inside of the cheek process. Note that the rostral part of the process is filled with mandibular mesoderm (mm) and the caudal part with the endodermal first pharyngeal pouch (p1). No crest cells are visible on the surface of the mandibular mesoderm. C. Transverse section of an embryo in which DiO has been injected into the core of the cheek process and DiI into the neural crest. Note that DiI-labeled cells are mostly scattered in the superficial part of the head or in the surface ectoderm (arrowheads), whereas DiO-labeled cells are in the core (mesoderm) part of the cheek process (arrow). nt, neural tube. D and E. Two days after DiI injection into the mandibular mesoderm. Two examples are shown as whole mounts. In D, labeled cells are distributed in the upper lip (ulp), and in E, labeled cells are also seen dorsal and posterior to the upper lip. F-I. Four days after DiO injection into the mandibular mesoderm. Four examples are shown as whole mounts. Labeled cells are distributed in various domains of the upper lip and mandibular-arch derivatives. Note the similarities in labeling patterns between D and H, and between E and I.





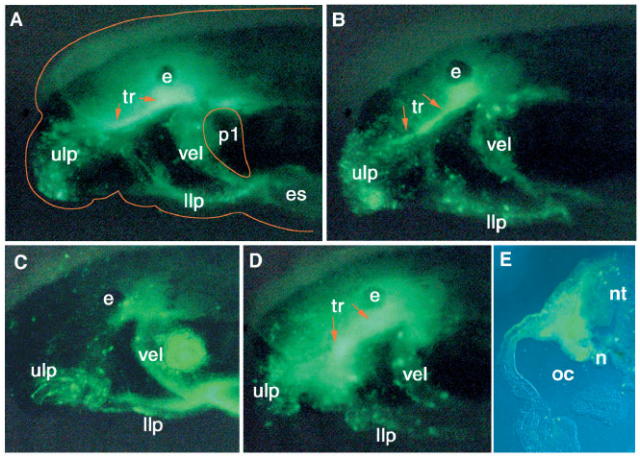


Fig. 3. Dye injections into the lamprey mandibular mesoderm II. Embryos are shown as whole mounts 7 days after DiI injections. Left lateral views except for E, which was sectioned transversely. A–D. Four embryos are shown as whole mounts. In three embryos (A, B, and D), labeling is heavy in the trabecular primordium (tr; red arrows) beneath and rostral to the eye (e). A fibrous pattern of labeling is seen in the upper lip (ulp) of all the embryos shown here. Labeling is also visible in the velum (vel) and the lower lip (llp), in a pattern similar to the muscle morphology. E. Transverse section of the embryo shown in A. Labeling is heavy in the mesenchyme lateral to the notochord (n), corresponding to the site of trabecula development. oc, oral cavity; p1, pharyngeal pouch 1; nt, neural tube.

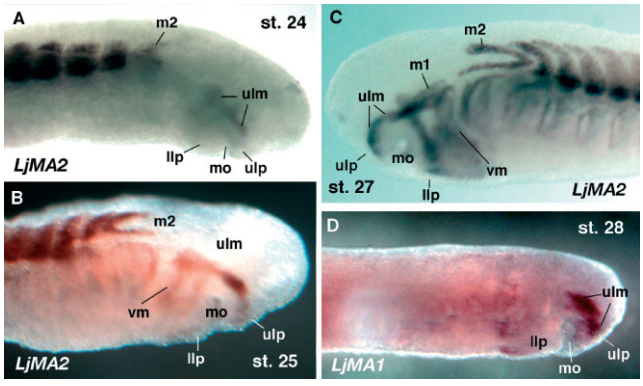


Fig. 4. Expression patterns of *LjMA* genes. A-C. Expression of *LjMA2* was detected by whole-mount in situ hybridization in a developing series of the lamprey. A. At stage 24, initial expression of *LjMA2* is in the oral region. High levels of expression are seen in the upper lip muscle (ulm), which is located in the superficial portion of the cheek process, spanning the upper lip (ulp) and the dorsal part of the mandibular arch. B. In the mandibular arch, the velar muscle (vm) is visualized by *LjMA2* gene expression. C. The upper lip muscle is now restricted within the upper lip. The first myotome (m1) has secondarily migrated rostrally, covering the upper lip and lower lip muscles laterally. *LjMA2* expression at this stage delineates the basic anatomy of the lamprey muscles. D. *LjMA1* expression at stage 28; the expression is restricted to the upper lip muscle. *llp,* lower lip; *mo*, mouth; *m2*, second myotome.

methanol at −20°C. Specimens were treated overnight with a 1:5 mixture of hydrogen peroxide and methanol, and were rehydrated in PBS containing 0.1% Tween 20 (PBT). After treatment with 0.2 N HCl in PBT for 10 min at RT, the samples were digested with 10 mg/ml proteinase K (Sigma). They were postfixed for 20 min with 4% PFA in PBT containing 0.2% glutaraldehyde, then washed with PBT, and prehybridized in hybridization buffer (50% formamide, 5 × saline sodium citrate [SSC], 1% sodium dodecyl sulfate [SDS], 0.05 mg/ml total yeast RNA, 50 mg/ml heparin sulfate, 5 mM ethylene diaminetetraacetic acid [EDTA]-Na_2, 0.1% CHAPS) for 1 h at 65°C. The specimens were then incubated in hybridization buffer with 0.1 mg/ml DIG-labeled RNA probe for 48 h at 65°C. After hybridization, the specimens were washed twice in 50% formamide, 5 × SSC, and 1% SDS for 30 min at 65°C, and the solution was substituted gradually with 10 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl and 0.1% Tween 20 (TST). RNaseA was added to a final concentration of 0.05 mg/ml and the specimens incubated for 30 min at RT. The samples were washed twice with 2 × SSC in 50% formamide for 30 min at 65°C, twice in 2 × SSC containing 0.3% CHAPS for 30 min at 65°C, and twice in 0.2 × SSC containing 0.3% CHAPS for 30 min at 65°C. For immunological detection, the embryos were blocked with TST containing 0.5% blocking reagent (Roche) for 60 min, and incubated with alkaline phosphatase (AP)-conjugated anti-digoxygenin Fab fragment (diluted 1:4000; Roche) at 4°C overnight. The specimens were washed five times for 60 min each in TST at RT. Alkaline phosphatase activity was detected with nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) in NTMT (Roche).

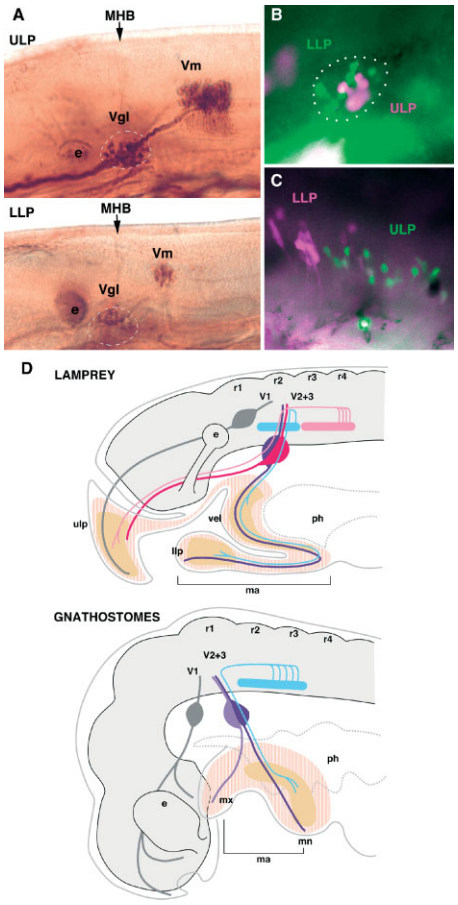
RESULTS

Labeling the lamprey mandibular mesoderm

In the early pharyngula of the lamprey around stage 21 (Tahara, '88), a pair of obvious protrusions has developed on both sides of the rostral head. These structures are called ''cheek processes'' (Damas, '44), and consist of expanding mandibular mesoderm and the first pharyngeal pouch endoderm (Fig. 2A, B; see also Kuratani et al., '99, 2001). It is only at later stages that the premandibular mesoderm develops into this process, as the lateral growth of the prechordal plate (Kuratani et al., '99). Furthermore, at this stage of development the migrating crest cells have not arrived at this level (Horigome et al., '99). Therefore, it is possible to label only the mandibular mesoderm at this stage by injecting either DiI or

Fig. 5. Innervation and morphological patterns of the trigeminal nerves. A–C. Results of upper lip (ulp) and lower lip (llp) labeling in lamprey larvae. With upper-lip labeling, the ventral part of the maxillomandibular ganglion (Vgl) and the posterior part of the trigeminal motoneurons (Vm) are labeled, and with lower-lip labeling, the dorsal part of the ganglion and anterior motoneurons are labeled. Sensory and motor somatotopies are shown in B and C, respectively. Dots in B indicate the contour of the maxillomandibular ganglion. e, eye; MHB, mid-hindbrain boundary. D. Comparison of trigeminal-nerve patterning in lampreys and gnathostomes. Both in the lamprey and gnathostomes, the trigeminal nerve consists of the rostral part called the ophthalmic nerve (V1) and the caudal maxillomandibular nerves (V2+3). In the lamprey, the dorsal component of the maxillomandibular nerve is distributed in the upper lip located in the premandibular part of the head. Sensory neurons innervating this area occupy the caudal part of the maxillomandibular ganglion, which is not the case in the gnathostomes. The mandibular-mesoderm-derived upper lip (ulp) muscles are innervated by the caudal motoneurons in the hindbrain (pink in D). The caudal component of the maxillomandibular nerve innervates the original mandibular-arch derivatives (ma), which consist of the velum (vel) and lower lip (llp). In gnathostomes, the oral apparatus differentiates from the mandibular arch, where the maxillomandibular part of the trigeminal nerve is distributed. Note that the somatotopy of the sensory neurons in the ganglion is the reverse of that in the lamprey, and that r4 does not contain any trigeminal motoneurons. Also, note that the gnathostome maxillomandibular-oral system is composed of a subset of the elements seen in the lamprey. e, eye; MHB, mid-hindbrain boundary; mn, lower jaw; mx, upper jaw; ph, pharynx; r1-4, rhombomeres.

Fig. 6. Schematic representation of the developmental pattern of the lamprey oral apparatus. Top: early embryonic pattern in which the cheek process contains only the mandibular mesoderm (mm) and the first pharyngeal pouch (p1) surrounded by the trigeminal crest cells (TC). Middle: in the cheek process at the following stage, the premandibular mesoderm (pmm) secondarily arises rostral to the mandibular mesoderm, as the initial anlage of the lamprey upper lip (ulp). Thus, the upper lip belongs to the premandibular domain (pm) of the lamprey head. Into this domain, the dorsal subset of the mandibular mesoderm grows to form the upper lip muscle. Here, the mandibular-arch domain (ma) is defined as the region occupied by the original mandibular mesoderm. Below: completed ammocoete oral apparatus is shown. The main part of the mandibular mesoderm differentiates in situ into the muscles of the velum (vel) and the lower lip (llp), as well as the trabecula of the lamprey, which is equivalent to the gnathostome parachordal cartilages that develop lateral to the notochord (not shown).



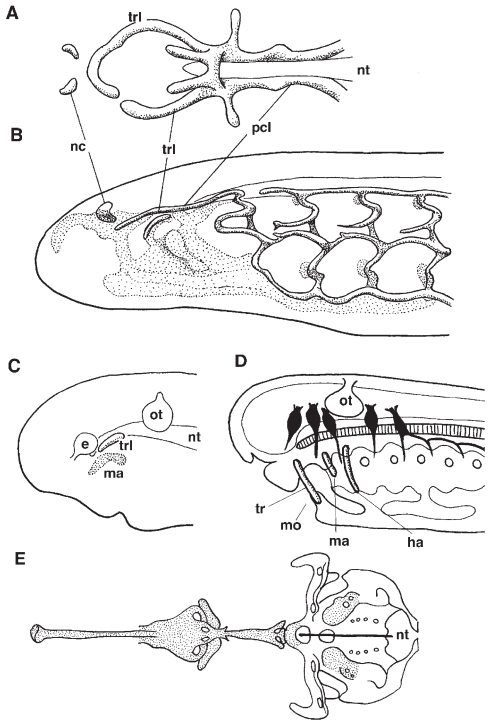
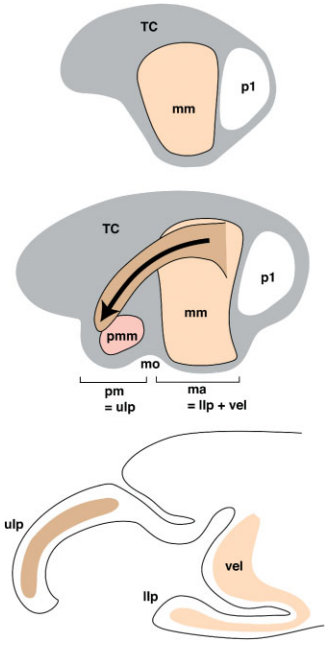


Fig. 7. Comparative morphology of the ''trabecula''. **A** and **B**. Morphology of the ammocoete cranial base by Johnels ('48). Ventral (**A**) and left lateral (**B**) views. The trabecula of the lamprey (trl) is a pair of cartilage rods beneath the brain, connected rostrally by a commissure cartilage at the base of the upper lip. This cartilage continues into the parachordal cartilage (pcl) posteriorly. At this stage, the lamprey trabecula is mostly prechordal in position. nc, nasal capsule; *nt*, notochord. **C**. Early development of the lamprey trabecula. The early primordium of the lamprey trabecula is located at the level of the notochord and mandibular-arch cartilage (ma). e, eye; ot, otic. A–D. Redrawn from Johnels (1948). **D**. Explanation of the ammocoete larva as an intermediate stage in the evolution of the gnathostomes, by de Beer ('37). Note that this author tried to find the trabecula in the perioral region, which does not represent the rostralmost part of the pharyngeal endoderm. ha, hyoid arch. **E**. Concept of the ''prechordal cranium'' proposed by Couly et al. ('93). Neural-crest-derived portion of the avian skull (stippled) is found rostral to the level of the notochord (nt). A large portion of the prechordal cranium differentiates from the paired 'true' trabecular cartilages that develop rostral to the notochord. Redrawn from Couly et al. ('93).



DiO fluorescent dye into the core of this process. For example, by injecting DiO into the cheek processes of embryos that had already been injected into the premigratory crest with another dye, DiI, we found that the presumptive mandibular mesoderm was selectively labeled with DiO, except for a small number of cells in the surface ectoderm. In contrast, a few DiI-labeled cells were found in a superficial position in the embryonic

head, apparently representing the cephalic crest cells (Fig. 2C).

We injected the dyes into the stage 21 mandibular mesoderm, and observed the distribution of labeled cells 2, 4, and 7 days after injection (Figs. 2, 3). For example, two days after DiI injection, when the cheek process had begun to develop a rostral process or the primordium of the upper lip, the labeled cells were distributed in the cheek-process derivatives, including the upper lip (Fig. 2D, E and data not shown). This pattern is consistent in embryos observed four days after injection (Fig. 2F-I). In several cases, a superficial longitudinal strand of cells was labeled, which was also observed in an identical pattern in embryos two and four days after injection (compare Fig. 2E and 2I). Seven days after injection (approximately stage 30; Fig. 3), labeled fibrous structures were apparent in the upper and lower lips, as well as in the velum, representing the trigeminal-nerve-innervated muscle of the ammocoete larva (Fig. 3 A–D; see below). High levels of labeling were often associated with cells located on both sides of the notochord (Fig. 3E), corresponding to the trabecular cartilage of this animal, according to the nomenclature of Johnels ('48).

Expression patterns of genes encoding muscle actin

Embryonic lamprey cranial muscles can be confirmed by several different methods. By histochemical analysis of acetylcholine esterase activity, and immunostaining for tropomyosin, the fibrous structures labeled in the stage 30 embryos and described above were found to be very similar to the trigeminal-nerve-innervated muscles of this animal (data not shown; see Kuratani et al., '97b, '99). In the present study, we found that members of the lamprey muscle actin gene family *(LjMA1* and *LjMA2)* were specifically expressed in subsets of lamprey muscles and their precursors.

The expression patterns of *LjMA2,* an early marker of muscle development, were examined by whole-mount in situ hybridization (Fig. 4A–C; a full description of the genes and their expression was published in Kusakabe et al., 2004). This gene is first expressed in the myotomes at stage 22, and its initial expression in the cranial muscles was observed at stage 24, when a low level of transcript was identified in the mesenchyme of the cheek process, in the shape of the early upper lip muscle, and the rest of the trigeminal-nerve-innervated muscle group (Fig. 4A). At stage 25, presumptive myoblasts formed two distinct masses of cells: the dorsolateral one that corresponds to the upper lip muscle, and the dorsoventrally extending medial mass that later differentiates into the velar and lower lip muscles in the mandibular-arch domain (Fig. 4B). By stage 28, the basic anatomical configuration of the lamprey trigeminal muscles was delineated by visualizing the expression of this gene (Fig. 4C). The distribution and appearance of LjMA2-positive cells at this stage resembled those of the DiO-labeled cells shown in Fig. 3. Therefore, considering these data together with the results of dye-labeling experiments, it is likely that the presence of the upper lip muscles in the lamprey larvae is due to the secondary migration of the subset of mandibular mesoderm cells that were primarily within the mandibular-arch domain, into the premandibular domain established in the later embryo.

On the other hand, another member of the *LjMA* gene family, *LjMA1,* is expressed in a more restricted subset of cranial muscles. According to a previous study, this gene is regarded as a marker for upper lip muscle (Fig. 4D), and in the cheek process of a stage 21 embryo, where no differentiation of the upper lip is visible, *LjMA1* expression is upregulated only in the dorsal part of the mandibular mesoderm (Kusakabe et al., 2004). Therefore, the upper lip muscle might be specified even before the morphological differentiation of the cheek process into the upper lip and the mandibular-arch subdivisions in the early mandibular mesoderm, by the specific upregulation of *LjMA1.* The latter hypothesis requires confirmation with further labeling studies, which will form part of our future research.

Sensory and motor innervation by the larval lamprey trigeminal nerves

The trigeminal-nerve-innervation pattern of the developing lamprey was detected by the application of dextrans conjugated with different fluorescent dyes or with biotin into scars made in the upper lip and mandibular-arch derivatives, including the lower lip and the velum (Fig. 5). It was not possible to label the lower lip and the velum separately. To evaluate the sensory innervation, labeling was examined in the trigeminal ganglion (see Johnston, '05 and Kuratani et al., '97b), and to evaluate the motor innervation, intramedullar labeling was examined at the level of the rostral hindbrain.

When the dextrans were applied to the upper lip domain, labeled cell bodies were seen in the ventroposterior subset of the trigeminal ganglion, and in the posterior part of the trigeminal motor nucleus (Fig. 5A–C). The application of dextran to the lower lip or velum resulted in the labeling of ganglia and motoneurons in a complementary fashion: the dorsorostral part of the trigeminal ganglion and the rostral motoneurons were labeled (Fig. 5A–C). Such labeling patterns are consistent with the description by Song and Boord ('93) and Koyama et al. ('87). According to a previous observation, the upper-lip-innervating motoneurons appear to occupy a region corresponding to r4, which does not contain trigeminal motoneurons in gnathostomes (Fig. 5D; based on the expression of the *LjKrox-20* and *LjPax6* genes; see Murakami et al., 2004).

DISCUSSION

Due to technical difficulties, the developmental dynamics of the lamprey head mesenchyme has not been extensively studied. The precise morphological distribution and premigratory mapping of the cephalic crest cells have been described with scanning electron microscopy and whole-mount in situ hybridization of marker gene expression, as well as with DiI labeling (Horigome et al., '99; Kuratani et al., '99; McCauley and Bronner-Fraser, 2002). However, no mapping data are available for the organogenetic development of the mesoderm or ectomesenchyme in the lamprey. Classical descriptions in comparative embryology are highly speculative, especially regarding the developmental fate of the mesoderm (Neal, 1897; Koltzoff, '01; Damas, '44). The only available data presented so far were derived from neural crest ablation experiments by Langille and Hall ('88), which showed the contribution of crest cells to the lamprey branchial cartilages (also see Newth, '51, '56) and the neural crest specification along the neuraxis, using DiI (Horigome et al., '99; Shigetani et al., 2002; McCauley and Bronner-Fraser, 2002).

In the present study, the developmental patterning of the mandibular mesoderm was first observed experimentally, using cell labeling, examination of gene expression patterns, immunostaining, and the pattern of trigeminal axonogenesis. It has often been exemplified in model animals, that the patterns of axonogenesis tend to follow the migration and distribution of the mesenchymal components (Johnston, '66; Noden, '75; Kuratani, '97). Our major findings are as follows. Firstly, labeling the lamprey mandibular mesoderm almost always results in the labeling of upper lip, lower lip, and velar muscles, as well as the mesenchymal condensation that represents the trabecular primordium lateral to the notochord. Secondly, the labeling pattern in the presumptive muscle primordium is identical to that observed with histological techniques and gene expression patterns (Figs. 3, 4). Thirdly, the expression pattern of a member of the muscle-actin-encoding gene family, *LjMA2,* is consistent with the predicted migration of the mandibular-mesoderm-derived muscle primordium into the premandibular domain (Fig. 4). Lastly, labeling the developing trigeminal neurons suggests the peculiar nature of the upper lip domain, compared with the patterns known in gnathostome development (Fig. 6). In the following discussion, evolutionary and developmental establishment of the lamprey upper lip will be addressed mainly in the context of evolutionary developmental biology and comparative embryology, to show that the observed developmental patterns are consistent with our previous hypothesis of jaw evolution, and finally to propose the evolutionary scenario of the biting jaw. In this context, the developmental identity as well as the origin of the cartilage called the trabecula will also be discussed.

Composite origin of the upper lip

In all the vertebrate embryos so far studied, a continuous large ectomesenchyme called the ''trigeminal crest cells'', can be found in the rostral head, filling the mandibular arch and the more rostral region, called the premandibular region (Fig. 1). The name of the trigeminal ectomesenchyme stems from the fact that the pattern of its distribution parallels that of the trigeminal nerve branches (reviewed by Kuratani et al., 2001). Therefore, the trigeminal crest cells can be anteroposteriorly subdivided into mandibular and premandibular domains, as the trigeminal nerve is subdivided into the ophthalmic and maxillomandibular portions (compare Figs. 1 and 5D). In gnathostomes, both the upper and lower jaws develop from the mandibular domain, corresponding to the distribution of axons belonging to the second (maxillar) and third (mandibular) branches of the trigeminal nerves (Fig. 5D; see Kuratani and Horigome, 2000; Kuratani et al., 2001). Three major branches are also recognized in the trigeminal nerve of the lamprey, including

the ophthalmic nerve homolog (Fig. 5D). Of these, the posterior two branches, innervating the upper lip and the velum + lower lip regions, have often been called maxillar and mandibular nerves, respectively (Johnston, '05; Kuratani et al., '97b; also see Goodrich, '30 for general description). Therefore, the patterning of the trigeminal nerve parallels that of the trigeminal ectomesenchyme, and therefore the nomenclature and homology of the nerve branches are tightly linked to the evolution of the jaw.

Our previous studies have shown that the patterning of the oral apparatus from the trigeminal ectomesenchyme proceeds through epithelial interactions between the mesenchyme and the surface ectoderm. In particular, the distribution of growth factors plays a role in the prepatterning of the oral apparatus (Shigetani et al., 2000; Fig. 1B).

However, the distribution pattern of FGF8, which prefigures the central part of the mouth opening, differs between the lamprey and gnathostomes, resulting in a heterotopic shift in the epithelial-mesenchymal interactions (Fig. 1B). Therefore, the homology between the ammocoete lips and gnathostome jaws is refuted; and at the same time, the developmentally innovative nature of the gnathostome jaw is confirmed (Kuratani et al., 2001; Shigetani et al., 2002). In terms of the initial distribution of the ectomesenchyme, the lamprey upper lip is identical to the premandibular derivatives of the gnathostomes, such as the nasal region and the trabecula (Figs. 1 and 5; see Kuratani et al., 1997a).

If the ammocoete upper lip has a premandibular identity, how can it possess trigeminal-nerve-innervated muscles? Experiments labeling the mandibular mesoderm of the early lamprey embryo, before the cheek process has differentiated into the upper lip anlage or the premandibular domain, indicate that a part of the mandibular mesoderm secondarily grows anteriorly and laterally and migrates into the upper lip domain (Figs. 2–4, 6). No such muscles are known in the gnathostomes, in which all the trigeminal-nerve-innervated muscles are restricted to derivatives of the upper and lower jaws.

It is not only the migration and gene specifications that are peculiar in the lamprey upper lip muscles. These muscles also appear to be innervated by a subset of trigeminal motoneurons located in r4 and rostral to the embryonic hindbrain (Fig. 5). This rhombomere is a segment of the hindbrain that never develops trigeminal motoneurons in gnathostomes (Murakami et al., 2004 and references therein). The craniofacial sensory innervation by the lamprey trigeminal nerve also appears peculiar because the somatotopic organization of the trigeminal ganglion cells is reversed relative to that of the gnathostomes (Arvidson, '77; Gregg and Dixon, '73; Martin and Dolivo, '83; Noden, '80; Scott and Atkinson, '99). Therefore, the lamprey upper lip stands out as a truly exceptional morphological and embryological unit in the head of vertebrates. The gnathostome oral apparatus appears to be constructed only from the mandibular component of the lamprey cheek process, consistent with the previous hypothesis (FGF8 and BMP4 signaling cascades are restricted to the mandibular domains of gnathostomes, see Fig. 1B; Shigetani et al., 2002).

For the reasons described above, it is unreasonable to call the upper-lip-innervating branch of the lamprey trigeminal nerve the ''maxillary nerve''. When compared with the developmental pattern of the oral regions and the hindbrain in the gnathostomes, the lamprey appears to possess a developmental program that does not exist in the gnathostomes, which includes the upper lip patterning mechanism, the rostrally migrating subset of mandibular mesoderm, and the upper-lip-innervating neurons (posterior part of the trigeminal ganglion and motoneurons developing in r4). If the gnathostome jaw has been acquired as an apomorphic character, as is generally accepted, these evolutionary processes might have proceeded as the loss of the developmental module, described above, in the ancestor of the gnathostomes. On the contrary, it is possible that the developmental pattern in the gnathostomes represents the plesiomorphic state, irrespective of the fossil record (reviewed by Janvier, '96). In this case, the upper-lip-related developmental mechanism should be regarded as newly invented in the lamprey system, through the heterotopic shift of epigenetic interactions.

Comments on the homology of the trabecula

In the present study, labeling the mandibular mesoderm resulted in the labeling of the dense mesenchyme below the brain that corresponds to the site of lamprey trabecular development (Fig. 3). In histological sections, the labeled cells were also found lateral to the notochord, similar to the site of the lamprey trabecula (Figs. 3E, 7B,C; Johnels, 1948). Such labeling patterns were observed consistently (eight of 12 successfully

labeled embryos, 67%), as was the case with the labeling of muscle tissues. Therefore, it is highly likely that this element actually represents the trabecular cartilage of the lamprey, and that this cartilage is derived from a mesodermal component, the mandibular mesoderm.

Historically, the morphological nature of the lamprey trabecula has been enigmatic (Fig. 7). This cartilage consists of a pair of rods beneath the brain, which are connected rostrally by a transverse commissure at the base of the upper-lip-derived oral hood of the ammocoete larva, thus providing the supporting tissue of the upper lip (see Johnels, 1948, for anatomy; Fig. 7A, B). The resemblance between this structure and the gnathostome trabecula is obvious: both develop as a pair of rods beneath the brain and are united rostrally with their counterparts (see Kuratani et al., 2001). An important difference between them, however, is that the lamprey trabecula develops at the level of the mandibular arch beside the notochord, whereas in the gnathostome, the trabecula develops prechordally (Fig. 7C). For this reason, Johnels ('48) refuted the homology between these cartilages, if they are collectively called the ''trabecula''. De Beer ('31, '37) also refused to call this cartilaginous ring in the lamprey the ''trabecula'', but for another reason (Fig. 7D). For de Beer, the trabecula had to be a premandibular structure, and he tried to find the lamprey homolog of this cartilage in the upper lip (Fig. 7D). As far as the morphological identity of the ectomesenchymal domains is concerned, the latter idea is very close to our present conclusion (also see below).

The prechordal position of the gnathostome trabecula implies its neural crest origin and also its chondrification in a notochord-free environment. The concept of ''prechordal cranium'' proposed by Couly and colleagues (1993) perfectly fits the embryonic development of the gnathostome trabecula, which actually differentiates into the part of the cranium called ''prechordal'' (Fig. 7E). The data presented in the present study, as well as the description by Johnels ('48), strongly suggest that what used to be called the lamprey 'trabecula' really has a mandibular mesodermal origin, and most likely corresponds to the parachordal cartilage of the gnathostomes, which extends rostrally to the level of the forebrain (Fig. 7A–C). Such a rostral position can be explained as the result of secondary elongation, because the initial position of the trabecula is found at the level of the mandibular arch (Johnels, '48). This is perfectly consistent with the results of our labeling experiments (Fig. 7C).

For the reasons described above, the lamprey homolog of the gnathostome trabecula, i.e., the premandibular and prechordal part of the neurocranium, is found in the ectomesenchyme of the upper lip. As the result of a heterotopic shift in ectoderm-ectomesenchyme interactions (involving FGF8 and BMP4), this part of the lamprey ectomesenchyme, which surrounds the premandibular mesoderm, has differentiated into a shape that resembles the gnathostome upper jaw (Shigetani et al., 2002), again consistent with the present labeling data.

ACKNOWLEDGMENTS

We thank Dr. Kiyokazu Agata and Yasuyo Shigetani for their technical suggestions and valuable discussion. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan (Grant numbers: 11309010, 11NP0201, and 11152225) to S.K.

LITERATURE CITED

Arvidson B. 1977. Retrograde axonal transport of horseradish peroxidase from cornea to trigeminal ganglion. Acta Neuropathol. (Berl). 38:49–52.

Boorman CJ, Shimeld SM. 2002. Cloning and expression of a Pitx homeobox gene from the lamprey, a jawless vertebrate. Dev. Genes Evol. 212:349–353.

Carroll RL. 1988. Vertebrate Paleontology and Evolution. W.H. Freeman and Co., New York.

Couly GF, Coltey PM, Le Douarin NM. 1993. The triple origin of skull in higher vertebrates: A study in quail-chick chimeras. Development 117:409–429.

Damas H. 1944. Recherches sur le développment de *Lampetra fluviatilis* L. contribution à l'étude de la cephalogénèse des vertébrés. Arch Biol Paris 55:1–289.

de Beer GR. 1931. On the nature of the trabecula cranii. Quart. J Microsc Sci 74:701–731.

de Beer GR. 1937. The Development of the Vertebrate Skull. Oxford University Press, London.

Gegenbaur C. 1898. Vergleichende Anatomie der Wirbeltiere mit Berücksichtung der Wirbellosen. Verlag von Wilhelm Engelmann, Leipzig.

Glover JC. 1995. Retrograde and anterograde axonal tracing with fluorescent dextran amines in the embryonic nervous system. Neurosci Protocols 30:1–13.

Goodrich ES. 1930. Studies on the Structure and Development of Vertebrates. McMillan, London.

Gregg JM, Dixon AD. 1973. Somatotopic organization of the trigeminal ganglion in the rat. Arch. Oral Biol 18:487–498.

Gregory WK. 1933. Fish skulls: A study of the evolution of natural mechanisms. Am Philos Soc 23:75–481.

Haeckel E. 1875. Die Gastrea und die Eifurchung der Thiere. Jena Z Naturwiss 9:402–508.

Hall BK. 1998. Evolutionary Developmental Biology, 2nd Ed. Chapman & Hall, London.

Horigome N, Myojin M, Hirano S, Ueki T, Aizawa S, Kuratani S. 1999. Development of cephalic neural crest cells in embryos of *Lampetra japonica,* with special reference to the evolution of the jaw. Dev Biol 207:287–308.

Janvier P. 1996. Early Vertebrates. Oxford Scientific Publications, New York.

Jarvik E. 1980. Basic Structure and Evolution of Vertebrates, Vol. 2. Academic Press, New York.

Johnels AG. 1948. On the development and morphology of the skeleton of the head of Petromyzon. Acta Zool 29:139–279.

Johnston JB. 1905. The cranial nerve components of Petromyzon. Morphol Jahrb 34:149–203.

Johnston MC. 1966. A radioautographic study of the migration and fate of neural crest in the chick embryo. Anat Rec 156:143–156.

Koltzoff NK. 1901. Entwicklungsgeschichte des Kopfes von Petromyzon planeri. Bull Soc Nat Moscou 15:259–289.

Koyama H, Kishida R, Goris RC, Kusunoki T. 1987. Organization of sensory and motor nuclei of the trigeminal nerve in lampreys. J. Comp Neurol 264:437–448.

Kuratani S. 1997. Distribution of postotic crest cells in the chick embryo defines the trunk/head interface: Embryological interpretation of crest cell distribution and evolution of the vertebrate head. Anat Embryol 195:1–13.

Kuratani S. 2003. Evolution of the vertebrate jaw—homology and developmental constraints. Paleontol Res 7:89–102.

Kuratani S, Horigome N. 2000. Development of peripheral nerves in a cat shark, Scyliorhinus torazame, with special reference to rhombomeres, cephalic mesoderm, and distribution patterns of crest cells. Zool Sci 17:893–909.

Kuratani S, Matsuo I, Aizawa S. 1997a. Developmental patterning and evolution of the mammalian viscerocranium: Genetic insights into comparative morphology. Dev Dyn 209:139–155.

Kuratani S, Ueki T, Aizawa S, Hirano S. 1997b. Peripheral development of the cranial nerves in a cyclostome, *Lampetra japonica:* Morphological distribution of nerve branches and the vertebrate body plan. J Comp Neurol 384:483–500.

Kuratani S, Horigome N, Hirano S. 1999. Developmental morphology of the cephalic mesoderm and reevaluation of segmental theories of the vertebrate head: Evidence from embryos of an agnathan vertebrate, *Lampetra japonica.* Dev Biol 210:381–400.

Kuratani S, Nobusada Y, Horigome N, Shigetani Y. 2001. Embryology of the lamprey and evolution of the vertebrate jaw: Insights from molecular and developmental perspectives. Phil Trans Roy Soc 356:15–32.

Kusakabe R, Takechi M, Tochinai S, Kuratani S. 2004. Lamprey contractile protein genes mark different populations of skeletal muscles during development. Mol Dev Evol 302B:121–133.

Langille RM, Hall BK. 1988. Role of the neural crest in development of the trabeculae and branchial arches in embryonic sea lamprey, *Petromyzon marinus* (L). Development 102:301–310.

Mallatt J. 1996. Ventilation and the origin of jawed vertebrates: A new mouth. Zool J Linn Soc 117:329–404.

Martin X, Dolivo M. 1983. Neuronal and transneuronal tracing in the trigeminal system of the rat using the herpes virus suis. Brain Res 273:253–276.

McCauley DW, Bronner-Fraser M. 2003. Neural crest contributions to the lamprey head. Development 130: 2317–2327.

Murakami Y, Ogasawara M, Sugahara F, Hirano S, Satoh N, Kuratani S. 2001. Identification and expression of the lamprey *Pax6* gene: evolutionary origin of the segmented brain of vertebrates. Development 128:3521–3531.

Murakami Y, Pasqualetti M, Takio Y, Hirano S, Rijli F, Kuratani S. 2004. Segmental development of reticulospinal and branchiomotor neurons in the lamprey: insights into evolution of the vertebrate hindbrain. Development 131:983–995.

Neal HV. 1897. The development of the hypoglossus musculature in *Petromyzon* and *Squalus*. Anat Anz 13: 441–463.

Newth DR. 1951. Experiments on the neural crest of the lamprey embryo. J Exp Biol 28:247–260.

Newth DR. 1956. On the neural crest of the lamprey embryo. J Embryol Exp Morphol 4:358–375.

Noden DM. 1975. An analysis of the migratory behavior of avian cephalic neural crest cells. Dev Biol 42:106–130.

Noden DM. 1980. Somatotopic organization of the embryonic hick trigeminal ganglion. J Comp Neurol 190:429–444.

Scott L, Atkinson ME. 1999. Compartmentalisation of the developing trigeminal ganglion into maxillary and mandibular divisions does not depend on target contact. J Anat 195:137–145.

Shigetani Y, Nobusada Y, Kuratani S. 2000. Ectodermally derived FGF8 defines the maxillomandibular region in the early chick embryo: epithelial mesenchymal interactions in the specification of the craniofacial ectomesenchyme. Dev Biol 228:73–85.

Shigetani Y, Sugahara F, Kawakami Y, Murakami Y, Hirano S, Kuratani S. 2002. Heterotopic shift of epithelial-mesenchymal interactions for vertebrate jaw evolution. Science 296:1319–1321.

Song J, Boord RL. 1993. Motor components of the trigeminal nerve and organization of the mandibular arch muscles in vertebrates. Acta Anat 148:139–149.

Steinberg M. 1957. A nonnutrient culture medium for amphibian embryonic tissues. Carnegie Institution of Washington Year Book 56:347–348.

Tahara Y. 1988. Normal stages of development in the lamprey, *Lampetra reissneri* (Dybowski). Zool Sci 5:109–118.

Wagner GP, Müller GB. 2002. Evolutionary innovations overcome ancestral constraints: A reexamination of character evolution in male sepsid flies (Diptera: Sepsidae). Evol Dev 4:1–6.