# Craniofacial Development and the Evolution of the Vertebrates: the Old Problems on a New Background

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**[REVIEW]**

# Craniofacial Development and the Evolution of the Vertebrates: the Old Problems on a New Background

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**ABSTRACT**—Based on recent advances in experimental embryology and molecular genetics, the morphogenetic program for the vertebrate cranium is summarized and several unanswered classical problems are reviewed. In particular, the presence of mesodermal segmentation in the head, the homology of the trabecular cartilage, and the origin of the dermal skull roof are discussed. The discovery of the neural-crest-derived ectomesenchyme and the roles of the homeobox genes have allowed the classical concept of head segmentation unchanged since Goethe to be re-interpreted in terms of developmental mechanisms at the molecular and cellular levels. In the context of evolutionary developmental biology, the importance of generative constraints is stressed as the developmental factor that generates the homologous morphological patterns apparent in various groups of vertebrates. Furthermore, a modern version of the germ-layer theory is defined in terms of the conserved differentiation of cell lineages, which is again questioned from the vantage of evolutionary developmental biology.

**Key words:** neural crest, craniofacial development, evolution, cephalic mesoderm

## A brief history of craniofacial studies

Craniofacial development and its evolution have long been an intriguing issue of vertebrate morphology. Interest in the subject initially began in the field of comparative osteology, with the question: Is there an archetype with segments in the skull? The number of segments incorporated into the skull was also an issue of debate that subsequently persisted as the central question of comparative embryology (reviewed by Goodrich, 1930; de Beer, 1937; Jarvik, 1980; Jefferies, 1986). The 'problem of head segmentation' was another name for the 'head problem' (Kopfprobleme). As first stated by Goethe and his colleagues, the vertebrate skull was perceived as an assemblage of vertebrae as found in the postcranial trunk, and early scientists tried to describe the cranium as a unified pattern consisting of an invariable number of vertebrae ('vertebral theory' of the skull; Fig. 1; Goethe, 1790; reviewed by Gaupp, 1898; Owen, 1866; reviewed by Goodrich, 1930; de Beer, 1937; Neal and Rand, 1946; and by Kuratani, 2003).

In the era of comparative embryology during the transi-

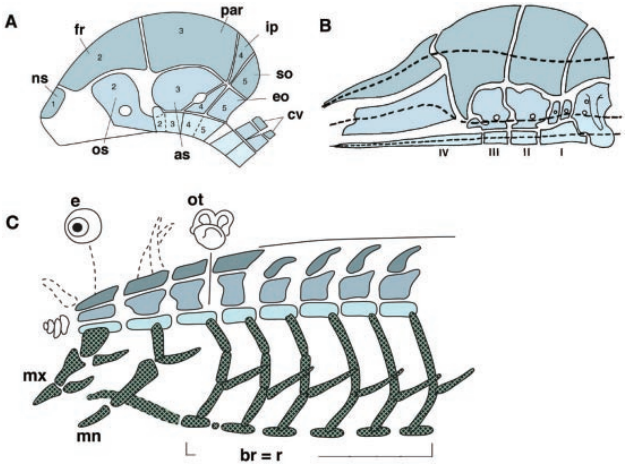


**Fig. 1.** Vertebral theories of the skull. (A) The vertebral theory proposed by Goethe based on the mammalian cranium. Five vertebrae are assumed in the skull. (B) A view by Oken. Four segments are counted; also based on the mammalian cranium. (C) The archetype proposed by Owen (1866). Unlike the current understanding, upper and lower jaws ('mx' and 'mn', respectively) are thought to represent two tandem branchial arches (br) that are further compared with the ribs (r) in the trunk. *Abbreviations:* as, alisphenoid; cv, cervical vertebrae; e, eye; eo, exoccipital; ip, interparietal; os, orbitosphenoid; ot, inner ear; par, parietal; so, supraoccipital.

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tion from the nineteenth to the twentieth century, the question of head segmentation again became a central topic. This was, at least in part, stimulated by the discovery of 'head cavities' in the shark embryo (Fig. 2; see Gee, 1996; and Kuratani, 2004a), appearing as mesodermal coeloms (Balfour, 1878; see below), as well as by the segmental origin of the occipital cartilage (see below). Head cavities are actually the origins of extrinsic eye muscles (somatic-muscle-like skeletal muscles in the trunk), and they appeared to arise segmentally, typically as three pairs, each associated with a single pharyngeal arch (PA), the ventral visceral element. The cavities were thus designated from anterior to posterior, the premandibular, mandibular, and hyoid cavities, innervated by the oculomotor, trochlear, and abducens nerves, respectively, in the same way that each myotome is innervated by spinal nerves in a segmental fashion (van Wijhe, 1882; reviewed by Goodrich, 1930; de Beer, 1937; Jarvik, 1980; Jefferies, 1986; Fig. 2). Thus, the vertebrate head was understood as an array of segments, each consisting of a dorsal somatic part and a ventral visceral part, just as the trunk consists dorsally of segmented somites, and ventrally of unsegmented lateral plate (the source of visceral smooth muscle).

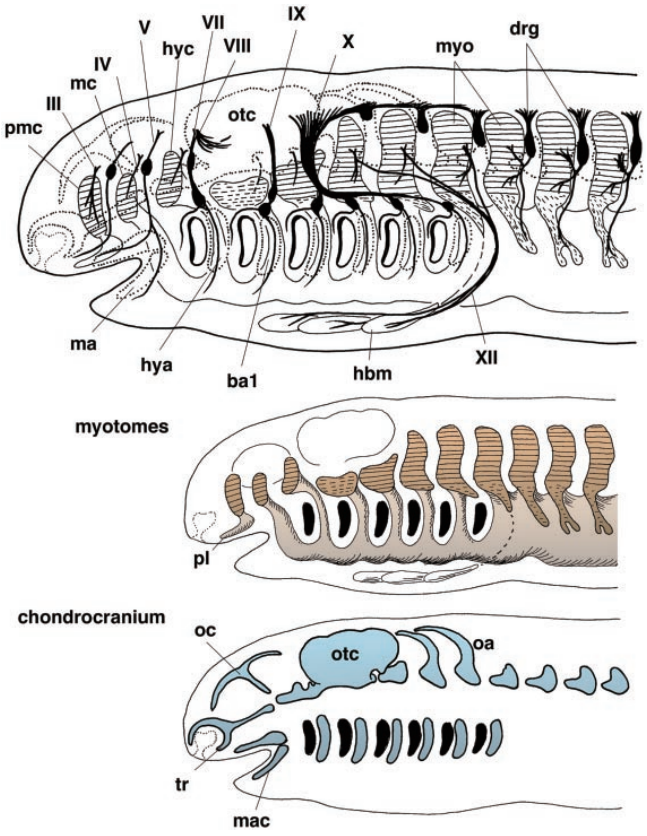


**Fig. 2.** Evolutionary embryological view of head segmentation. This scheme was originally drawn by Goodrich (1930) based on shark embryos. It has been redrawn with its mesodermal and chondrocranial elements extracted below. Note that Goodrich understood the vertebrate (gnathostome) cranium to be a segmentally organized structure, made of mesodermal segments equivalent to the somites in the trunk, connected ventrally by pharyngeal arches that are repeated at the same intervals as the mesodermal segments.

The cranium was also understood in terms of the same scheme as was applied to head segmentation. Generally, the vertebrate cranium was divided dorsoventrally into the neurocranium, or the capsule that supports the brain, and the viscerocranium, which supports the pharynx (Gaupp, 1906; Goodrich, 1930; Gregory, 1933; de Beer, 1937; Portmann, 1969: Fig. 3). Because the viscerocranium is segmented into the units of the pharyngeal (branchial) arch skeletons (e.g., Sewertzoff, 1911), the neurocranium was also thought to be segmented, and the pilar cartilages between the cranial nerve roots were often equated with the neural arches of the vertebrae (Gaupp, 1906; de Beer, 1937; Starck, 1980; reviewed by Kuratani, 2003). Thus, the vertebrate skull was explained as having a single shared morphological pattern, secondarily modified by animal-group-specific variations and differentiation, as seen in mammalian-specific traits such as the middle ear ossicles, the malleus, incus, and stapes, derived from the articular, quadrate, and hyomandibular respectively of less derived animals.

The morphological scheme of the skull described above was defined primarily functionally, as the supporting tissues for the brain and pharynx (see Gregory, 1933; Fig. 3), although the concept of homology does not necessarily require the preservation of ancestral functions (Owen, 1866). Therefore, the anterior part of the neurocranium, the derivative of the trabecular cartilages, was thought to represent another pair of pharyngeal cartilages, belonging to another pharyngeal arch once present in the ancestor (reviewed by de Beer, 1931, 1937; but see Kuratani *et al*., 1997, 2004). This hypothesis fits well with the head segmentation theory, which involves the premandibular segment, as well as the trigeminal nerve, as the composite cranial nerve; the ophthalmic nerve was assumed to belong to the premandibular segment and the maxillomandibular nerve to the mandibular segment (reviewed by Goodrich, 1930; de Beer, 1937; Jarvik, 1980). Thus, early comparative studies focused on assigning each cranial element to a common segmental scheme, without necessarily questioning the developmental mechanisms involved. Although the developmental origins of structures were often the focus of debate, the mainstream concept was mesodermal segmentation. Although the involvement of the neural-crest-derived ectomesenchyme in craniogenesis had been pointed out (Platt, 1893), it does not seem to have affected the morphological scheme of the cranium as described above (see Goodrich, 1930 and de Beer, 1937).

With the advent of experimental embryology, the developmental role of the crest-derived ectomesenchyme in vertebrate craniogenesis, and the importance of tissue-tissue interactions became generally accepted (reviewed by Gans and Northcutt, 1983; Northcutt and Gans, 1983; Hall and Hörstadius, 1988; Le Douarin, 1982; Le Douarin and Kal-

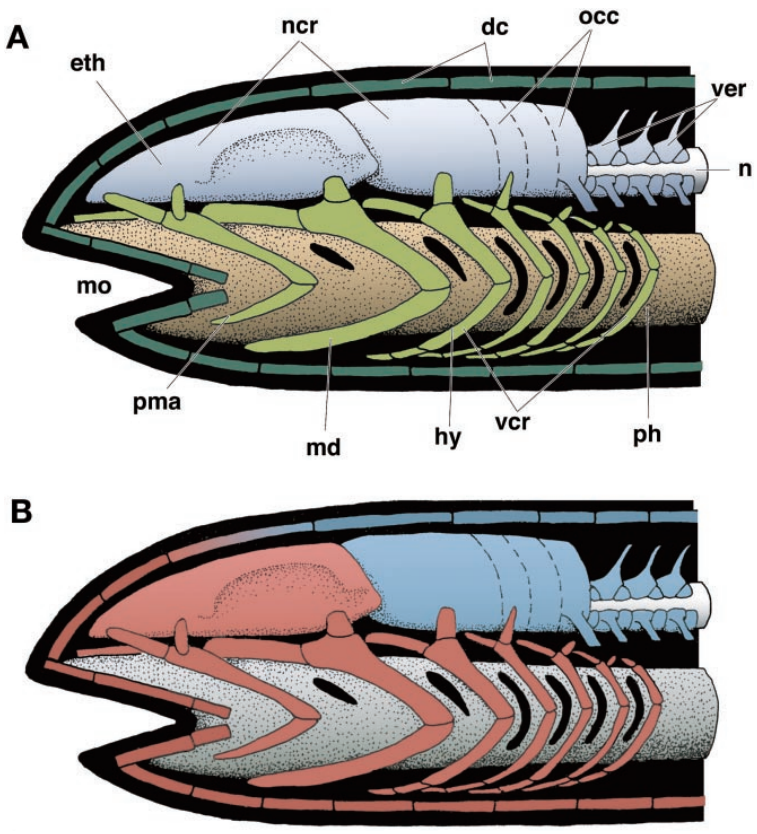


**Fig. 3.** Primary architecture of the vertebrate cranium, redrawn from Portmann (1969). (A) According to the original scheme, the vertebrate (gnathostome) skull is assumed to be composed of the cartilaginous neurocranium (light blue), the viscerocranium (light green), and the dermatocranium (dark green). This scheme reflects the functional properties of each cranial parts, although the distinction partly reflects the types of cell lineages and the developmental mechanisms that give rise to each component. The posterior half of the neurocranium corresponds to the mesodermally derived chordal cranium of Couly *et al.* (1993), and its caudal end, or the occipital bone, differentiates from several rostral somites in many gnathostome species. The dermatocranium is further subdivided into the neural- and visceral elements. Note that one visceral arch skeletal element (= hypothetical premandibular arch skeleton) is drawn rostral to the mandibular arch, which does not coincide with the presence of the ethmoid part of the neurocranium (= prechordal cranium) in this scheme, if the premandibular arch refers to the trabecula and its derivatives. (B) Neural-crest-derived elements have been colored red, and the mesodermal elements blue, based on several, cell-labeling and molecular genetic experiments reported by Le Lièvre and Le Douarin (1975), Le Lièvre (1978), Noden (1984), Couly *et al.* (1993), and Morriss-Kay (2001), and on the assumption that homologous skeletal elements are derived from identical cell lineages among species. There are several different opinions regarding the origin of the dermal skull roof (neural part of the dermatocranium), which may differ in each animal group. A number of studies have suggested that at least its most rostral part is derived from the neural crest. *Abbreviations: dc*, dermatocranium; *eth*, ethmoidal region of the neurocranium; *hy*, hyoid arch; *md*, mandibular arch; *mo*, mouth; *n*, notochord; *ncr*, neurocranium; *occ*, occipital; *ph*, pharynx; *pma*, premandibular arch; *vcr*, viscerocranium; *ver*, vertebrae or the vertebral column.

cheim, 1999; and by Hall, 1999; also see de Beer, 1926 for the mutual importance of experimental embryology and comparative embryology; and Hanken and Hall, 1993, for a modern treatment of the issue). However, this was never truly integrated with the transcendental morphology until recently, when molecular developmental biology became the glue to unite them (see de Beer, 1926, 1937, 1958; Jarvik, 1980; Couly *et al*., 1993, 1998; see Hanken and Hall, 1993, for studies of the vertebrate skull after de Beer, 1937).

Based on molecular genetics and experimental embryological techniques, current research into vertebrate craniogenesis and evolution focuses on the developmental mechanisms involved in the differentiation of the crest-derived ectomesenchyme, the regulatory mechanisms underlying coordinated expression patterns of various regulatory genes, including the *Hox* and *Dlx* genes, and the inductive signaling pathways that lead to the differentiation of specific cell populations. Evolutionary developmental biology (reviewed by Hall, 1998; Hall and Olson, 2003) has undoubtedly been influenced by this movement since the end of the last century, although some curious questions that arose in comparative embryology remain unanswered today. In the present review, I examine the possibility that these remaining questions can be dealt with in our modern understanding of craniofacial morphology, and argue that some of these are extremely important and relevant to the evolutionary developmental biology of the vertebrates. The new ideas of developmental biology have already shed light onto these topics, although this has rarely been discussed.

## Segments in the mesoderm – somitomerism

### *Preotic mesoderm*

The idea of head segmentation was more or less influenced by the transcendental or idealistic philosophy of classical morphology, or by its descendent, the comparative embryology, until the beginning of twentieth century. Those embryologists and anatomists believed that, even if segments were invisible in the adult skull, segmental material should still be visible in the primordial cranium (reviewed by Goodrich, 1930; de Beer, 1937). In fact, at the posterior end of the neurocranium (the postotic region) in many vertebrate groups, the occipital bone develops through the fusion of several postotic somite-derived sclerotomes. This was confirmed more precisely in a modern experiment using chicken and quail chimeras (Couly *et al*., 1993), because the quail cells have a unique nuclear marker discernible in chicken tissue. The preotic region, on the other hand, was more problematic, and provided stronger evidence in support of the (mesodermal) head segment theory: the discovery of head cavities in elasmobranch embryos. Thus, the problem of head segmentation can be divided into two parts, the preotic and postotic, corresponding to problems related to the cephalic mesoderm and somites in the embryo, respectively.

**Preotic region – the head cavities**

The evolutionary and developmental significance of the head cavities is still unclear (Figs. 2, 4, and 5). However, they can never be a primitive trait for all vertebrates because there are no head-cavity-like structures in lamprey or hagfish embryos, the most basal group of the vertebrates, if the head cavities are defined as true coelom lined with thin epithelium, floating in head mesenchyme that is composed mainly of loose connective tissue (fibroblasts) (for lamprey, see Kuratani *et al.*, 1999; see Koltzoff, 1901 and Damas, 1944, for classical descriptions). On the other hand, the head cavities have been described in most of the gnathostome taxa, and they appear to diminish in a caudal to rostral direction along the phylogenetic tree crownwards (Fig. 6).

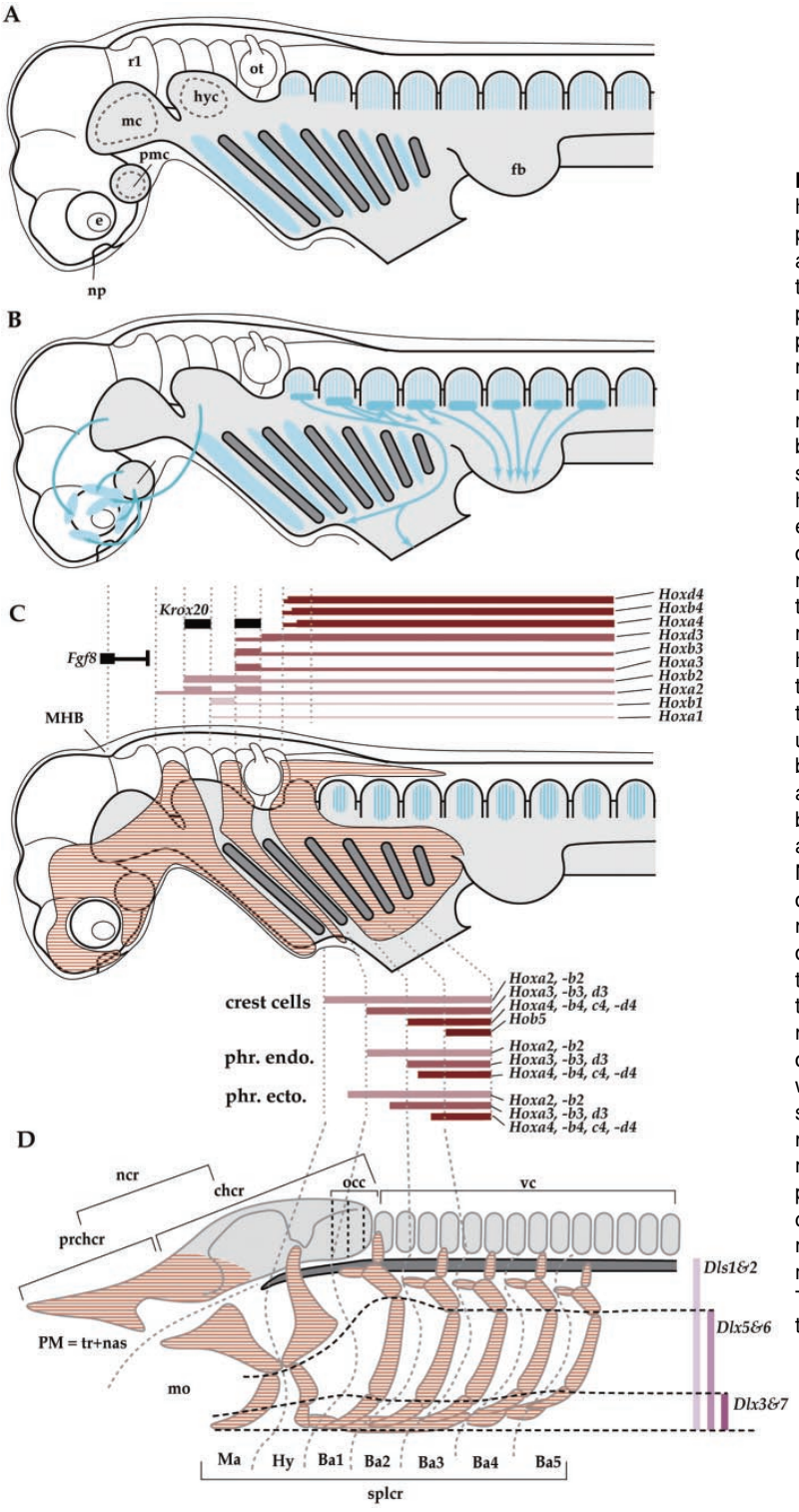


**Fig. 4.** Developmental configuration of the vertebrate head. (A) Mesoderm (shaded gray) is segmented only postotically, and the head mesoderm is unsegmented and regionalized only into pseudosegmental 'regions' that develop into the mandibular (mc), hyoid (hyc), and premandibular (pmc) cavities (or mesoderm), by the presence of other embryonic elements such as the pharyngeal pouches (gill slits) and otocyst (ot). Of the head mesodermal regions, the premandibular mesoderm may represent a true segment. Blue shading indicates the branchial arch muscle primordia, and blue hatching the skeletal muscle primordia. (B) Myoblasts. Three pairs of head mesodermal regions differentiate into six extrinsic eye muscles around the eye (e). Some of the somite-derived myotomal cells express the *Lbx1* gene, which is necessary for the ventral migration of these cells to form the hypobranchial and limb (fin) muscles. (C) Cephalic neural crest cells. The crest cells (hatched red) in the head migrate along the dorsolateral pathway to populate the PAs, thereby forming an extensive ectomesenchyme that can be divided into three parts. These crest cell populations are connected to the even-numbered rhombomeres of the hindbrain. In gnathostomes, *Hox* genes are expressed in nested, collinear patterns in the hindbrain and PAs, constituting positional values for the arches to differentiate into the appropriate morphology. Note that the mandibular arch is specified as a Hox-code default state. The *Hox* gene expression is believed to be repressed in the rostral part of the head, at least in part, due to the growth factor, FGF8, which is released from the midbrain-hindbrain boundary (MHB). (D) Gnathostome chondrocranium. The crest-derived part is colored red. The rest of the cranium and vertebral column are derived from the mesoderm (gray). The cranium as a whole is divided into the neurocranium (ncr), or the capsule for the brain, and the viscerocranium (splanchnocranium, splcr) that supports the pharynx. The neurocranium is further divided into the crest-derived prechordal cranium (prchcr) and the mesoderm-derived chordal cranium (chcr). Note that the posterior part of the neurocranium, or the occipital (occ), is primarily segmented because it is derived from some rostral somites. The prechordal cranium is believed to differentiate from the premandibular crest cells. *Abbreviations: fb*, fin bud.

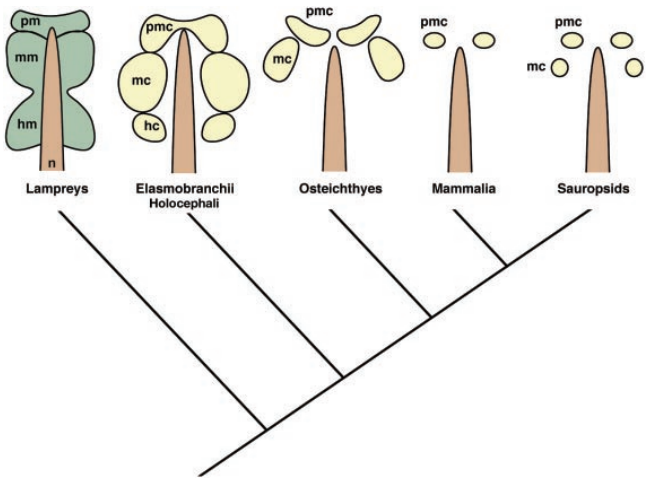


**Fig. 5.** Evolution of head cavities. The morphology of the head cavities is diagrammatically illustrated and plotted on the phylogenetic tree. Referenced data are as follows. Holocephalans: Dean (1906); elasmobranchs: Balfour (1878), Marshall (1881), van Wijhe (1882), de Beer (1924), Bjerring (1977), Jarvik (1980), Kuratani and Horigome (2000); osteichthyes: de Beer (1924), Kuratani *et al.* (2000); reptiles and birds: Oppel (1890), Adelmann (1926), Wedin (1949, 1953a,b); mammals: Fraser (1915), Gilbert (1947, 1953, 1954, 1957); also see Kuratani (2004a) for a review. In the lamprey, no real head cavities arise but the cephalic mesoderm is only 'regionalized' into domains that are arranged in a pattern homologous to that seen in the shark head cavities. The complete set of three head cavity pairs is only seen in elasmobranch and holocephali embryos. The animal species with epithelial head cavities occur crownwards on the phylogenetic tree, and head cavities can be regarded as a synapomorphy that defines the gnathostomes. Note that there is a tendency for the cavities to disappear in a posterior-to-anterior direction. The mandibular cavities in the non-mammalian amniotes are vestigial, if present. *Abbreviations: hm*, hyoid mesoderm; *mc*, mandibular cavity; *mm*, mandibular mesoderm; *n*, notochord; *pm*, premandibular mesoderm; *pmc*, premandibular cavity.

Therefore, head cavities do not seem to represent primitive characters in the vertebrates, although they may constitute a synapomorphy, with which to define the gnathostomes.

The developmental function of head cavities is still unknown as is their patterning mechanism. The concept of generative constraint is important for the segmental pattern in development: certain patterns established in the early embryo will affect subsequent patterning in a restrictive conserved manner, resulting in a shared anatomical pattern in different animal groups. A typical example is in the somites that pattern the dorsal root ganglia and spinal nerve roots. The trunk neural crest cell populations and motor nerve fibers are not initially segmented, but are secondarily subdivided by the presence of somites, resulting in the segmental pattern of the spinal nerves (Detwiler, 1934; Keynes and Stern, 1984; Tosney, 1988; reviewed by Kuratani, 2003). Therefore, the primary factor in certain patterns is the presence of primary segments. Do head cavities have the same segmental pattern as the cranial nerve roots and rhombomeres? The answer is no. In the shark embryo, although the cavities maintain topographical relationships with the nerves throughout development, the positions of the nerve roots shift rostrocaudally along the neuraxis of the hindbrain (Kuratani and Horigome, 2000). Moreover, the same morphological patterns of the cranial nerves occur in many gnathostome embryos, with or without overt epithelial cavities. Thus, the cavities do not seem to function as a generative constraint in the patterning of the peripheral nerves.

**Somitomeres or regionalization of the head mesoderm**

Another noteworthy pseudosegmental structure is the so-called 'somitomeres'. Unlike head cavities, which are primarily epithelial, somitomeres are mesenchymal and lack overt segmental boundaries. True somitomeres were originally observed by scanning electron microscopy as incomplete segmental bulges in the paraxial mesoderm of the trunk region prior to somitogenesis (Bellairs and Sanders, 1986). Similar bulges have often been recognized in the cephalic mesoderm of some vertebrate species, and were first called 'cephalic somitomeres' as opposed to those found in the trunk (Meier, 1979; Anderson and Meier, 1981; Meier and Tam, 1982; Meier and Packard, 1984; Jacobson, 1988, 1993 see Fig. 5). This series of reports has shown that, throughout vertebrate species, the cephalic mesoderm show a rather conserved topographical pattern relative to embryonic structures such as the otic placode and optic vesicle, and has stereotypical relationships with the crest cell streams. With this conserved morphological pattern, it seemed to be justified to give the same name to each region of the cephalic mesoderm. Unlike the clear anatomical pattern of the head cavities, however, the presence of somitomeres is problematic. According to the computer-assisted analyses of cell aggregations, there is no segmental pattern in the cephalic mesodermal cells (Freund *et al.*, 1996). At least, somitomeres cannot be equated with the head cavities that count less than half of the somitomeres. In comparative embryology too, the lack of a clear histological definition of mesodermal segments has given rise to various opinions regarding the number of head segments (reviewed by Kuratani, 2004a).

The problem in evaluating the mesodermal segments is twofold. Firstly, is there a remnant segmental pattern in the cephalic mesoderm that does not exert generative constraints segmentally upon other structures, like the somites pattern the crest cell streams? Secondly, do 'pseudosegments' reflect any ancestral developmental program at all?

In response to the first question, the head mesoderm does not impose generative constraints on any other embryonic tissues to create cranial anatomical patterns. Even if the somitomeres represent a remnant segmental pattern, the morphological pattern of the vertebrate head is not segmented as the trunk shows metamerical patterns generated by somites. The rhombomeres and pharyngeal pouches, rather than the cephalic mesoderm, pattern the cranial nerve morphology (Kuratani and Eichele, 1993; Begbie *et al.*, 1999; Begbie and Graham, 2001).

In addressing the second problem, there are several

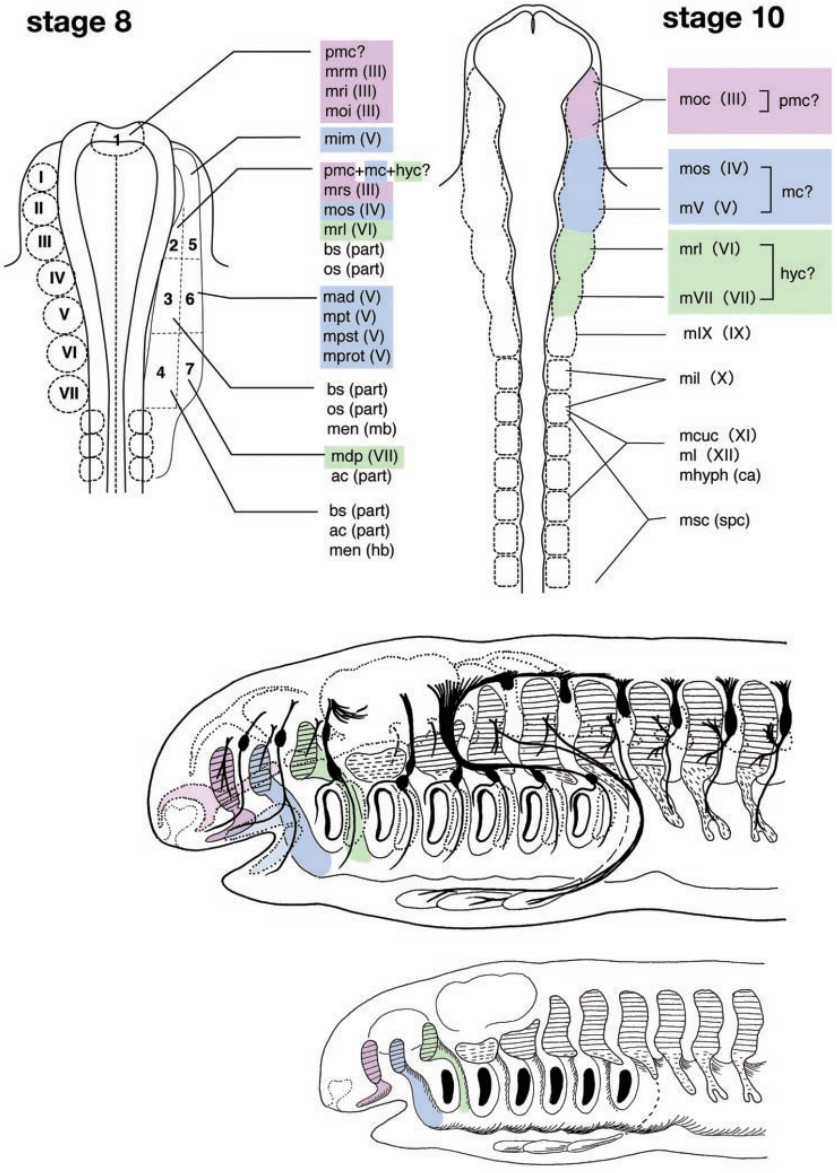


**Fig. 6.** Mapping the head mesoderm and the theory of head segmentation. Grades of specification and regionalization of the head mesoderm may differ at each stage of development. Top: Fate mapping data of Couly *et al.* (1992) and Noden (1988) are compared. The former is based on stage 8, and the latter on stage 10 chicken/quail embryos. Below: The segmented head proposed by Goodrich (1930) and its simplified form, in which a segmental association between the pharyngeal arches and extrinsic eye muscle primordia, or the head cavity is assumed. The premandibular, mandibular, and hyoid segment derivatives are colored according to this scheme. These colors are also applied to the mesodermal structures in avian embryos on the top. Note that fate mapping based on stage 10 chicken embryos is more organized segmentally when the grafting was performed at a later stage, reminiscent of the classical view of head segmentation. Such organization may reflect the regionalization of the initially 'unmapped' head mesoderm, which leads to the pattern of the pharyngula, not necessarily indicating that the head mesoderm is segmented as are the somites. Also see Borue and Noden (2004) for the fate mapping based on stages 8.5 to 9+ embryos. Interestingly, this mapping shows an intermediate result between the two shown in this figure.

reasons to refute an innate segmental program in the cephalic mesoderm of vertebrates. Specific mesodermal regions can be identified in a way comparable between animal species, not by the segmentation of the mesodermal cell mass, but by regionalization of the mesoderm into several domains by the presence of some other embryonic structures (Fig. 4A; see Kuratani *et al.*, 1999; Kuratani, 2003; also see Horigome *et al.*, 1999). For example, the mandibular mesoderm can be defined as a cell mass found in the mandibular arch (limited posteriorly by the first pharyngeal pouch), and the hyoid mesoderm is limited caudally by the otic placode and the second pharyngeal pouch and anteriorly by the first pharyngeal pouch. It is after this stage of regionalization that the cephalic mesoderm appears to be segmentally specified, as illustrated by segmentalists in comparative embryology like Goodrich (1930; see below and Fig. 6). Therefore, even if it is possible to morphologically distinguish specific region in the head mesoderm in a way that satisfies the concept of morphological homology among various species, it does not mean that there is a segmental pattern in the head mesoderm, similar to that found in the postotic somites.

Of the recognizable mesodermal 'regions', the premandibular mesoderm, which arises relatively late in development from the prechordal plate, and has a clear posterior boundary, may represent a real 'segment'. In a recent molecular analysis, oscillation of segment-related gene expression was observed only twice in chicken cephalic mesoderm, once in the premandibular mesoderm and once in the rest of the head mesoderm (Fig. 7; Jouve *et al.*, 2002). If the segmental compartment is defined by these molecular functions, this result suggests that the entire head mesoderm of vertebrates represents a single large segment, equivalent to a single somite. This idea does not support the hypothetical number of mesodermal segments assumed in the vertebrate ancestor (Holland, 2000), or that of somitomeres.

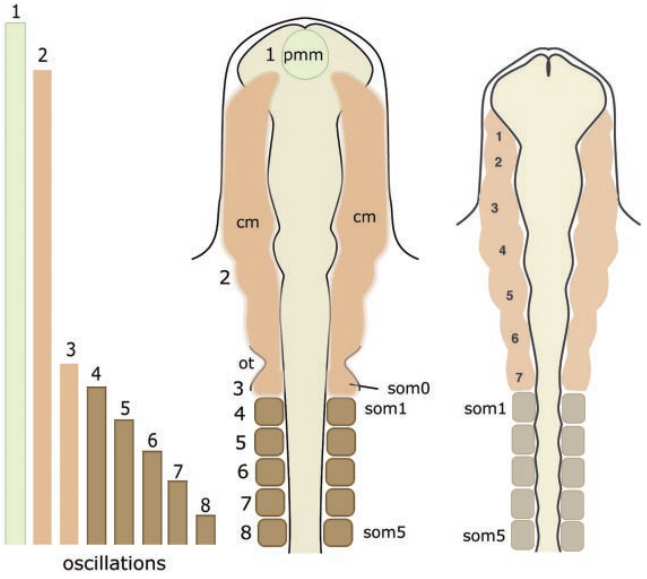


**Fig. 7.** Somitomeres and somitogenetic genes. The morphological pattern of somitomeres in the chick embryo is shown on the right, as a simplified illustration. Hypothetical somitomeres are numbered. On the left is shown the oscillating expression of a somitogenetic gene, *chairy*, in the early chick embryo, based on Jouve *et al.* (2002). Each oscillation is numbered together with the mesodermal part generated after that oscillation. Note that there are only two oscillations in the head mesoderm, one for the premandibular mesoderm, and the other for the rest of the cephalic mesoderm.

**Transposition and homeotic transformation**

As noted above, the posterior part of the neurocranium is developmentally segmented into somites (Figs. 3, 4). In comparative embryology, the occipital bone has been regarded as part of the original trunk, which was secondarily assimilated and integrated in craniogenesis. However, the number of occipital vertebrae differs among animal species, indicating that different numbers of segments can have the same morphological identity. Therefore, the morphological homology of skeletal elements cannot be reduced to a serial number of developmental compartments. Needless to say, this problem should be dealt with primarily in terms of the axial specification of the vertebral column, to which the coordinated expression patterns of homeobox-containing genes (*Hox* genes) are profoundly related (Fig. 8).

*Hox* genes encode transcription factors and are arranged tandemly on the DNA that constitutes *Hox* clusters. As the result of genomic duplications, there are four *Hox* clusters in amniote and basal vertebrate genomes, although the teleost clusters seem to have undergone another duplication event. *Hox* gene paralogues occupying equivalent positions in the clusters are paralogue group (PG) genes, which are numbered in the 3' to 5' direction as PG1, PG2, and so forth (McGinnis and Krumlauf, 1992).

In the developmental specification of skeletal elements based on the *Hox* genes, there is a tendency, called 'spatial colinearity', such that the *Hox* genes located more 3' within the clusters are expressed more anteriorly and those at the 5' end are expressed more posteriorly along the anteroposterior axis of the embryonic trunk (Fig. 8). Because these genes are usually expressed from certain anteroposterior levels posteriorly, each somite along the axis expresses a specific set of *Hox* transcripts with a nested pattern. This pattern of *Hox* gene expression is called the 'Hox code', and has been shown experimentally to function as a system conferring a positional value on the somite at each level, so that it can differentiate during development to its appropriate morphological identity (Kessel and Gruss, 1990, 1991; Kessel, 1992). Interestingly, the same morphological identities of the vertebrae, including the occipital, are encoded by homologous sets of *Hox* genes in all animal groups, not by the number of segments. Therefore, there seem to have been no somites added secondarily or lost during evolution, or morphological identities associated with the numbers of somites, as was assumed by several authors (Gegenbaur, 1887; Kastschenko, 1888; Fürbringer, 1897; Sewertzoff, 1895; Gaupp, 1898). However, a heterotopic shift in *Hox* gene regulation (establishment of the *Hox* code) appears to be the basis for the evolutionary transposition of vertebral identities, as was assumed by another group of embryologists (Rosenberg, 1884; Sagemehl, 1885, 1891; Goodrich, 1910, 1930). Therefore, the regulation of the Hox code probably changed through evolution, creating vertebral formulae that differ in each animal group (Fig. 8B). The same mechanism can of course explain the evolution of vertebral formulae, as stated by Burke and her colleagues (Burke *et al.*, 1995; reviewed by Narita and Kuratani, 2005 in press).

**Fabric of the cranium**

***Modern version of the germ-layer theory***

One of the major tasks of experimental embryology was to elucidate the history of cells that generate certain structures or organ systems; that is mapping studies based on clonal analyses. Even before this biological discipline entered the arena of morphology, there was a belief, based only on the observation of embryos, that morphologically homologous structures are derived from identical germ layers. This idea also stems from the idealistic embryology and is called the 'germ-layer theory' (von Baer, 1928).

The original version of the germ-layer theory was refuted by the discovery by experimental embryologists that the crest-derived ectomesenchyme contributes to the craniofacial skeletons, although this had been assumed well before it was confirmed by experimental evidence. As noted above, the mesodermal mesenchyme was believed to be the major source of the vertebrate skeleton, and the same importance was given to the head mesoderm as was attributed to the somites in the trunk. The placodal origin of some peripheral ganglia is another reason to refute the theory (reviewed by de Beer, 1958). However, we still tend to think that specific cell lineages are consistently utilized for a specific spectrum of cell types or skeletal components. In terms of modern developmental biology, therefore, the spirit of the germ-layer theory could be re-expressed as our inductive propensity that, 'morphologically homologous structures are

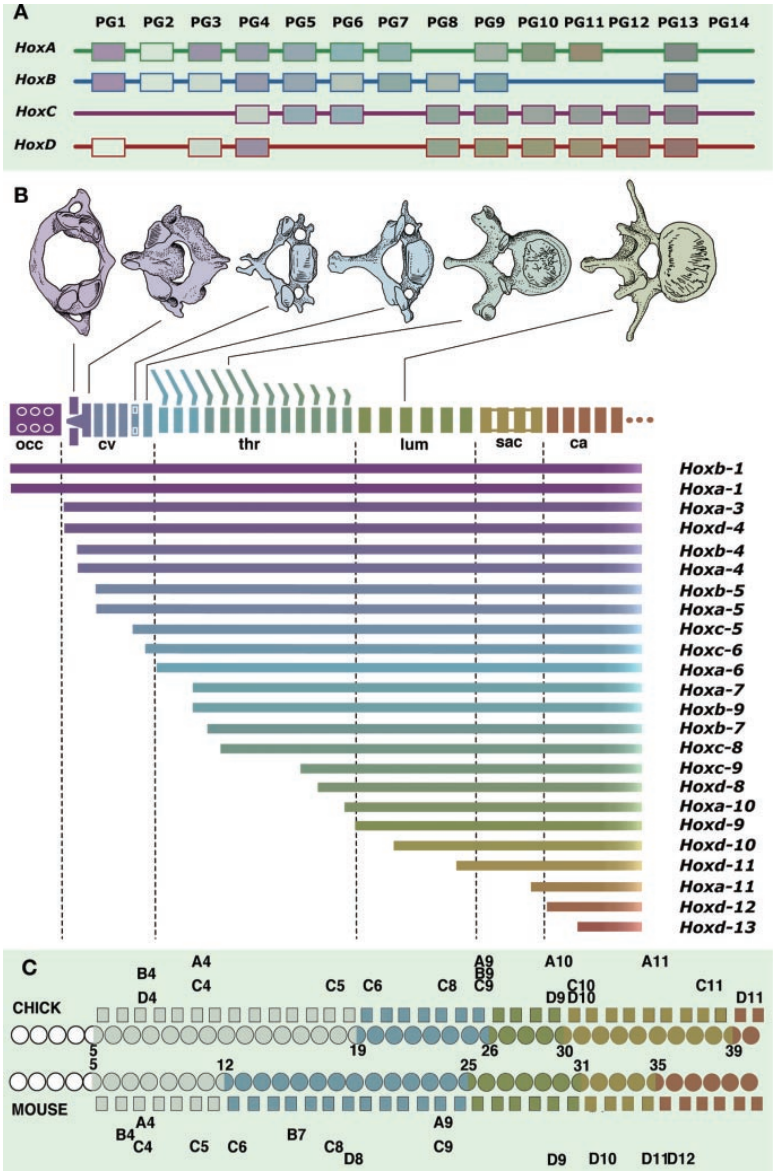


**Fig. 8.** Homeotic transformation of the vertebral column and *Hox* genes. (A) There are four *Hox* clusters (*HoxA* to *HoxD*) in the genomes of non-teleost gnathostomes. (B) Schematic illustration of Hox-code-dependent vertebral specification in the mouse, based on Kessel (1992). Each *Hox* gene is expressed with a slightly different rostral expression boundary in the trunk, largely based on a nested, collinear pattern. Thereby, the provertebra at each level is specified by a specific set of *Hox* genes, facilitating the axial specification of the vertebral column. Note that the occipital bone, the posterior part of the neurocranium, is also specified as part of the vertebral column derived from an array of somites. (C) Comparison of the Hox codes of the chicken and mouse, based on Burke *et al.* (1995). Note that morphological identities are not associated with specific numbers of somites but with the expression domains of identical (homologous) *Hox* genes. *Abbreviations: ca*, caudal vertebrae; *cv*, cervical vertebrae; *lum*, lumbar vertebrae; *occ*, occipital vertebrae; *thr*, thoracic vertebrae.

(or tend to be) produced from conserved and restricted cell lineages'.

Modern techniques such as vital dye labeling, the construction of chimeric embryos, and the discovery of crest cell-specific molecular markers, have clarified that the extensive crest-derived ectomesenchyme primarily occupies the ventral part of the vertebrate head, as opposed to the more axially and dorsally located head mesoderm (Figs. 4, 9, 10; Noden, 1988). This ventral ectomesenchyme is also seen in the lamprey (Horigome *et al.*, 1999; Takio *et al.*, 2004) and is suggested by histological observation in the hagfish embryo (von Kupffer, 1900). Several questions arise. Does the distinction of cell lineages (crest versus mesoderm) coincide with the anatomical configuration of the cranium (viscerocranium versus neurocranium)? Is this correlation conserved through evolution? In the modern version of the germ-layer (cell lineage) theory, the question must be asked: Is the morphological homology of the skull consistently derived from certain specific cell lineages through specific developmental mechanisms? If not, is there a more suitable morphological division of the skull that corresponds to the division of cell lineages or cell types, such as the mesoderm and crest cells?

## Neural crest versus head mesoderm

The neurocranium is located in a dorsal part of the head, encapsulating the central nervous system, whereas the viscerocranium supports the pharynx, with the pharyngeal arch skeletal complex (Fig. 3). Dermal exoskeletal, and cartilage-preformed endoskeletal parts are associated with both components (Fig. 3; for the evolutionary origin of the skeletal elements and neural crest, see Hall, 1999). It is generally accepted that the entire visceral skeleton is of crest origin (Figs. 3, 4, 10; Hall and Hörstadius, 1988; Le Douarin, 1982; Noden, 1983, 1988; Le Douarin and Kalcheim, 1999). Therefore, does the above neurocranial/viscerocranial dis-

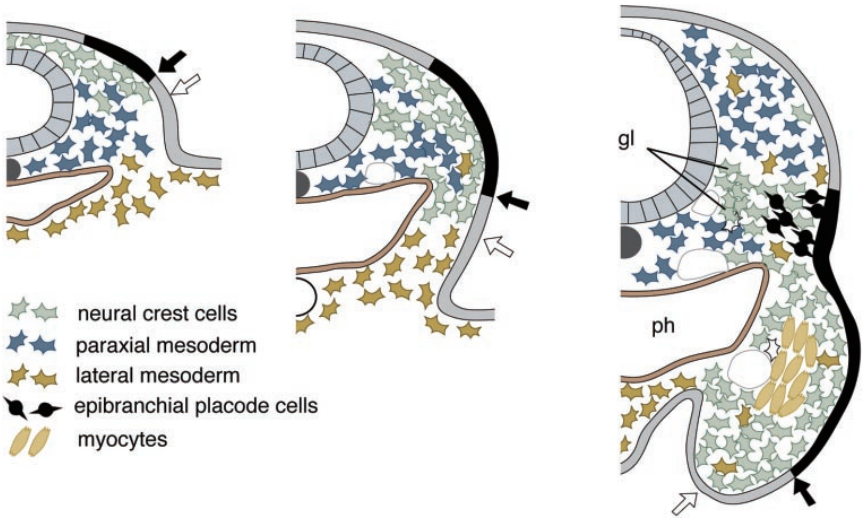


**Fig. 9.** Development of cephalic crest cells. The distribution pattern of cephalic neural crest cells in the developing chicken embryo is diagrammatically illustrated, based on Noden (1988). Arising at the junction of the neurectoderm and surface ectoderm, the crest cells in the head migrate ventrally and laterally along the superficial pathway, called the dorsolateral pathway. They finally occupy the PA, to form the ectomesenchyme surrounding the branchial arch muscle primordium derived from the cephalic mesoderm. Note that, at the latest stage, the crest-derived ectomesenchyme and mesoderm are dissociated dorsoventrally, and the future cranial base is composed of mesodermal cells lateral to the notochord. White arrows indicate the leading edge of the migrating crest cells, and the black arrows the relative growth of the cephalic surface ectoderm. Note where the position of the epibranchial placode is mapped in the young embryo. *Abbreviations: gl*, sensory ganglion of the branchial nerve; *ph*, pharynx.

tinction correspond to the embryonic distribution patterns and fates of the crest-derived ectomesenchyme and mesodermal mesenchyme?

As simply summarized by Noden (1988), most of the craniofacial structures are derived from crest cells, whereas the 'neurocranium' is partly of mesodermal origin. Of the neurocranial elements, the entire ethmoid (nasal capsule), a part of sphenoid bone, and a part of the otic capsule are made of neural crest cells. Couly *et al.* (1993) more precisely identified the distinction between the crest-derived and mesoderm-derived parts of the skull base at the level of the hypophysial foramen (Fig. 10A). This boundary corresponds to the site, at which the trabecular cartilages attach to the rostral end of the parachordal cartilage, or the ridge called the 'crista sellaris' in some amniotes (de Beer, 1937; the crista sellaris represents the posterior margin of the hypophysial foramen, and is not homologous to the dorsum sellae in mammals. The latter is a direct derivative of the orbital cartilage).

The extent of the mesodermal neurocranium corresponds to the rostral limit of the notochord. The notochord and cephalic mesoderm together end rostrally behind the adenohypophysis. Because the mesoderm requires notochord-derived signals to chondrify (Figs. 9–11), Couly *et al.* (1993) called the rostral, crest-derived part of the neurocranium the 'prechordal cranium', which can chondrify without induction by the notochord. Obviously, this embryonic distinction of the skull is based on the assumption that certain cell types constantly require the same inductive mechanisms. Therefore, the cranial sidewall and base can be divided into two portions corresponding to the presence and absence of the notochord, reflecting a difference in the origins of cells (crest or mesoderm), as well as a difference in the signaling mechanism that causes them to differentiate into skeletal tissues.

Although mesodermally derived skeletal elements are found in the region close to the notochord, Schneider (1999) found that when crest-derived ectomesenchyme is transplanted ectopically in place of paraxial mesoderm destined to form the orbitotemporal region, it can differentiate into skeletal elements that are morphologically indistinguishable from those normally generated by mesoderm. Thus, cell lineages can be interchangeable in certain limited developmental contexts irrespective of the classification of skeletal

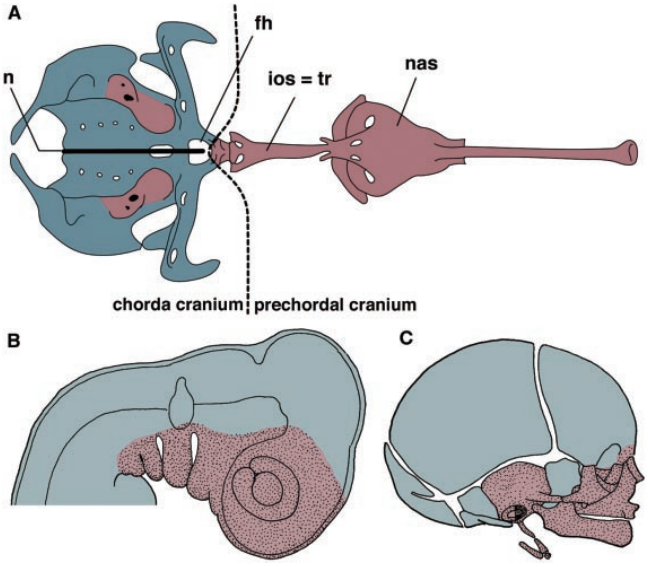


**Fig. 10.** Neural-crest-derived parts of the amniote cranium. (A) Origin of the 'neurocranium' based on experiments on avian embryos reported by Couly *et al.* (1993). The mesodermal part of the cranium is colored blue, and the crest-derived part red. Note that the interface between the mesodermal and crest-derived parts of the neurocranium corresponds to the hypophysial foramen (fh) or the rostral limit of the notochord (n). (B) Distribution of the cephalic mesoderm (blue) and crest-derived ectomesenchyme (red, stippled) in the chicken pharyngula by Noden (1988). Compare with Fig. 9. The ectomesenchyme occupies the ventral half of the head, including the PAs and the premandibular region. (C) Results of avian chimeric experiments were extrapolated to the human perinatal skull, based on Noden (1988). In his view, the cranial vault is thought to be of mesodermal origin. Abbreviations: *ios*, interorbital septum; *nas*, nasal capsule; *tr*, trabecula.

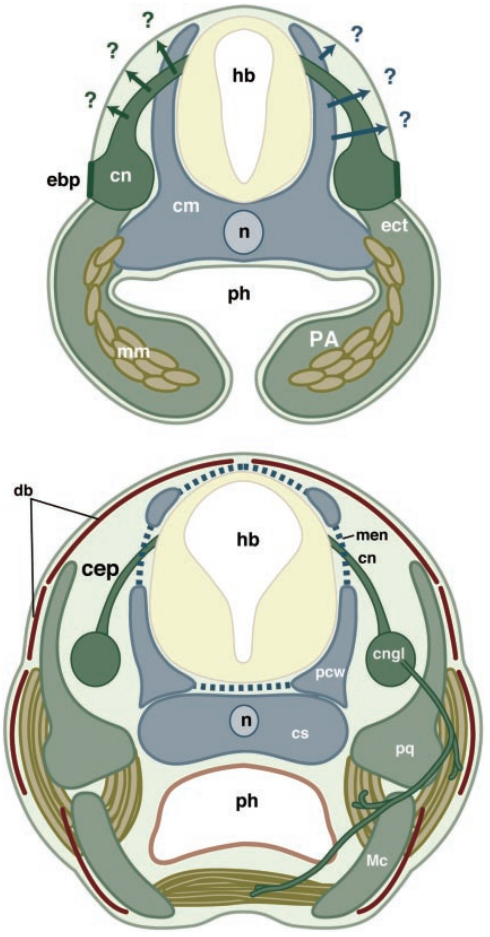


**Fig. 11.** Cell lineages and topography. Anatomical and embryonic configuration of the vertebrate cranium at the level of the mandibular arch is diagrammatically illustrated, based on Kuratani (2004a). The mesodermally derived neurocranium (blue) differentiates through induction by the notochord (n) and the neurectoderm (hindbrain; hb), and crest-derived cells form the cranial nerve (cn; dark green) and branchial skeletons (light green) in the pharyngeal arch (PA). In the completed cranium (below), the dermal bones (db; dark red) have developed in superficial layers. Note that the dermal bones in the skull roof can reasonably originate either from crest cells or from the cephalic mesoderm (arrows). *Abbreviations: cep*, cavum epiptericum; *cm*, cephalic mesoderm; *cngl*, cranial nerve ganglion; *cs*, central stem; *ebp*, epibranchial placode; *ect*, ectomesenchyme; *Mc*, Meckel's cartilage; *men*, meningeal membrane; *mm*, mandibular mesoderm; *pcw*, primary cranial wall derived from the cephalic mesoderm; *ph*, pharynx; *pq*, palatoquadrate.

elements to form. Moreover, crest-derived ectomesenchyme likely responds to similar cues that promote skeletogenesis and facilitate proper patterning of mesodermally-derived skeletal elements. In normal development too, each part of the otic capsule appears to chondrify through an identical induction mechanism if the capsule is composed of both crest cells and mesoderm (Noden, 1988; Couly *et al*., 1993). Therefore, the distinction between crest-specific and mesoderm-specific inductive signaling may be gratuitous in a strict sense, and terms such as 'neurocranium' and 'viscerocranium' may be primarily associated with the embryonic environment (places). Each cell type simply tends to populate specific positions in the embryo as the result of, for example, specific migration patterns of crest cells and the original distribution of the cephalic mesoderm, which are highly constrained during phylotypic stages (evolutionarily stabilized). Similar phenomena are also recognized for myogenic mesodermal cells. Although some myogenic gene expressions are cell-autonomously regulated, the morphological patterning of the cells is highly dependent on the embryonic environment (Borue and Noden, 2004). These phenomena apparently violate the modern version of the germ-layer theory. The implication of Schneider's experiment is that the generation of the vertebrate morphological pattern is largely dependent on epigenetic interactions, which are based on the topographically organized morphological pattern of the embryo, not entirely on the cell-lineage-associated programs.

**Origin of the dermal skull roof**

A problem remains regarding the 'crest versus mesoderm' scheme of the vertebrate cranium, in the origin of the dermal skull roof. According to the morphological concept, the dermocranium can be divided into visceral and neural components, and if the posterior part of the endoskeletal neurocranium is of mesodermal origin, then so is the dermal skull roof (Fig. 3). Therefore, by the early 1990s, this part of the dermal skull roof was believed to be of mesodermal origin. However, Couly *et al.* (1993) showed that these skeletal elements also originate from the neural crest. Although most of the ectomesenchyme occupies the ventral portion of the embryonic head, the sites at which the dermal elements differentiate correspond to the dorsolateral migratory pathway characteristic of the cephalic crest cells (Fig. 11). Accordingly, either crest- or cephalic mesoderm-derived cells could reasonably differentiate into these skeletal elements.

Recent analyses on transgenic mice have implied that there is an anteroposterior distinction in the dermal bones between those derived from the cells that once activated the *Wnt1* promoter (a possible lineage marker for neural crest cells), and those derived from cells that did not (Jiang *et al*., 2002; also see Morriss-Kay, 2001). This problem, which concerns the most superficially located skeletal elements, is still unresolved and is difficult to access regardless of the anatomical position of these elements. It is also possible that homologous dermal elements develop from different cell lineages in each animal group. Here again, topography would be the only factor imposing a developmental constraint, providing a clue to the morphological homology. In this context, the dermal bone homologies have been ascribed in aquatic species, to the morphology of the lateral line system (Jarvik, 1980; Starck, 1980). Like the patterning of the otic capsule, the dermal bone patterning also possibly may be an epigenetic event, dissociated from any specific cell lineage.

## Evolution and development of the viscerocranium

### *Cephalic Hox code and branchiomerism*

If PA skeletons are mutual serial homologs, how can they differentiate into specific morphologies appropriate to their positions? Noden (1983) showed that when the neural crest destined to populate the mandibular arch (PA1) was transplanted to the hyoid arch (PA2) level (approximately at the level of rhombomere 4) of the host, some skeletal elements in PA2 developed with mandibular identities, such as quadrate and articular, rather than as hyoid arch skeletal elements, such as the columella auris and retroarticular process, which are normally expected in this arch (Fig. 12A; see footnote).

Historically, the experiment of Noden described as above was a prelude to the studies of *Hox* gene functions in the PA system. The Hox code also functions in the PA ectomesenchyme. In all gnathostome embryos examined so far, the PG2 *Hox* gene is expressed in PA2 and posterior to it, the PG3 gene in PA3 and posterior to it and so forth (Fig. 4; Hunt *et al*., 1991a,b). There are no *Hox* genes expressed in PA1, and differentiation of the jaw appears to be specified by the absence of *Hox* transcripts in the ectomesenchyme, designated the 'Hox-code default state' in this arch (Rijli *et al*., 1993; Couly *et al*., 1998; see below and footnote). Recent analyses have shown that agnathans may share the same basic Hox code, consisting of PG2 and PG3 from PA1 through PA3 of the embryonic pharynx (Takio *et al*., 2004; Kuratani, 2004b; also see Cohn, 2002).

The developmental function of the 'cephalic Hox code' has been shown experimentally, at least, in the specification of PA2 morphology as opposed to PA1. The disruption of *Hoxa-2*, expressed in PA2 and posterior to it, leads to the transformation of PA2 to share partial identity with PA1 (Fig. 12; Rijli *et al*., 1993; Gendron-Maguire *et al*., 1993). In contrast, overexpression of *Hoxa-2* results in the transformation of PA1 into the identity of PA2 (Pasqualetti *et al*., 2000; Grammatopoulos *et al*., 2000). In these experiments, therefore, PA1 and PA2 appear to represent equivalent developmental units that can change their developmental fates when different positional values are experimentally imposed on them, implying a developmental basis for branchiomeric transformation.

The scheme of cephalic Hox code as above partly fits the classical concept of branchial arch transformation. The ancestral vertebrate used to possess a series of undifferentiated PAs, and each PA has gradually acquired its specific differentiation program through evolution, depending on its positional values. Unlike the situation seen in the evolution of the vertebral formulae, the regulation of the Hox code in the PAs does not seem have changed through evolution. Therefore, we can identify the equivalent arches of different animals with the same name, such as 'mandibular' and 'hyoid' quite consistently. Importantly, the ancestor with undifferentiated an series of PAs appears to be purely theoretical, and, as mentioned above, the mandibular, hyoid, and branchial arch identities appear to have been present already in the common ancestor of the lamprey and gnathostomes (Takio *et al*., 2004; Kuratani, 2004b). Moreover, the origin of the jaw appears to have involved a complicated shift in tissue interactions, not simply transforming the man-

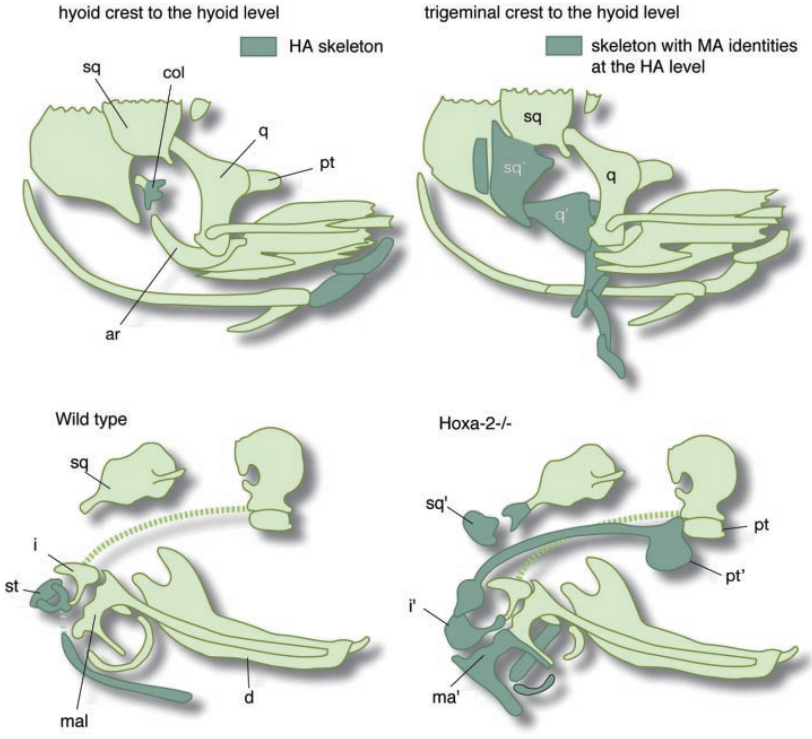


**Fig. 12.** Developmental specification of the branchial arch skeletons - two isomorphic experiments. Top: Based on the chick-quail chimeric experiment, Noden (1983) replaced the neural crest destined to become PA2 with crest ranging from the posterior midbrain to the rostral hindbrain (for PA1). On the left is the control experiment in which the PA2 crest in the chicken was isotopically replaced with that of quail. PA2-derived skeletal parts are populated with quail cells (hyoid-arch-derived skeletal elements). Note that the columella and part of the hyoid apparatus are derived from quail cells. On the left is the result of the heterotopic graft. In place of the hyoid arch skeleton (columella), those with mandibular arch identities are duplicated posterior to the endogenous PA1 skeletons. Bottom: *Hoxa-2* disruption in the mouse based on the experiment of Rijli *et al*. (1993). The wild-type embryonic skeleton is shown on the left. The stapes (homologous to the non-mammalian columella) and Reichert cartilage are the hyoid arch derivatives. In the *Hoxa-2* mutant on the right (as seen in Noden's experiment), ectopic skeletal elements with PA1 morphological identities are duplicated in place of PA2 skeletons. These two experiments are isomorphic, and both can be regarded as homeotic transformations of PA2 skeletons to the identity of PA1. *Abbreviations: ar*, articular; *col*, columella; *d*, dentary; *i*, incus; *i'*, duplicated incus; *ma'*, duplicated malleus; *mal*, malleus; *pt*, pterygoid; *pt'*, duplicated pterygoid; *q*, quadrate; *q'*, duplicated quadrate; *sq*, squamosal; *sq'*, duplicated squamosal; *st*, stapes.

dibular arch (Kuratani *et al*., 2001; Shigetani *et al*., 2002; reviewed by Kuratani, 2004a; also see Lee *et al*., 2004, and Cerny *et al*., 2004, for the development and evolution of the upper jaw; also see Janvier, 1996, 2003 for a paleontological review).

At any rate, the Hox code is no more than a developmental system with which to assign positional values to each of the arches, and the 'Hox-code default' does not necessarily mean the 'prototype of PAs' in any sense (see Fig. 13). The mandibular arch in vertebrates is usually the most highly diversified of all the arches. In comparative morphology, the prototypic PA morphology has been identified in the shape of postotic branchial arches, in which a certain number of cartilage elements are commonly identified in many gnathostomes, and even the shape of the mandibular arch skeleton may be regarded as a modified version of this pattern (Portmann, 1969; Jarvik, 1980; see Kuratani, 2004b). Still, this pattern does not represent the 'ancestral shape' of the arch skeleton, because no similar skeletal pattern has been found in agnathans.

**Jaw and trabecula**

The developmental meaning of the 'Hox-code default state' also remains unclear. As shown by the disruption of *Hoxa-2*, the duplicated morphological identity involved only the proximal part of the mandibular arch, and the Hox-code default state does not seem to pattern the more rostral portion of the mandibular arch skeleton. Another homeobox gene, *Otx-2*, may be responsible for the patterning of that region, because haploinsufficiency of this gene results in a spectrum of reductions in the proximal portion of PA1 (Matsuo *et al*., 1995; reviewed by Kuratani *et al*., 1997). In other words, both *Otx-2* expression and the Hox-code default state together pattern PA1 in a complementary fashion (reviewed by Kuratani *et al*., 1997). Interestingly, the expression patterns of the lamprey cognates of *Otx-2* and some *Hox* genes appear very similar to those of the gnathostomes (Ueki *et al*., 1998; Tomsa *et al*., 1999; Horigome *et al*., 1999; Takio *et al*., 2004), implying that this molecular coding for PA1 also was established very early in vertebrate history, whether the animals had developed the jaws or not. Whether these two domains are clear compartments, and where in the mandibular arch the boundary occurs, are questions that have not yet been answered, or even addressed.

More problematic is the presence of more rostrally located ectomesenchyme, also derived from trigeminal crest cells that do not express any *Hox* genes, rostral to the mandibular arch. This corresponds to the region where the 'prechordal cranium' of Couly *et al.* (1993) is assumed to differ-

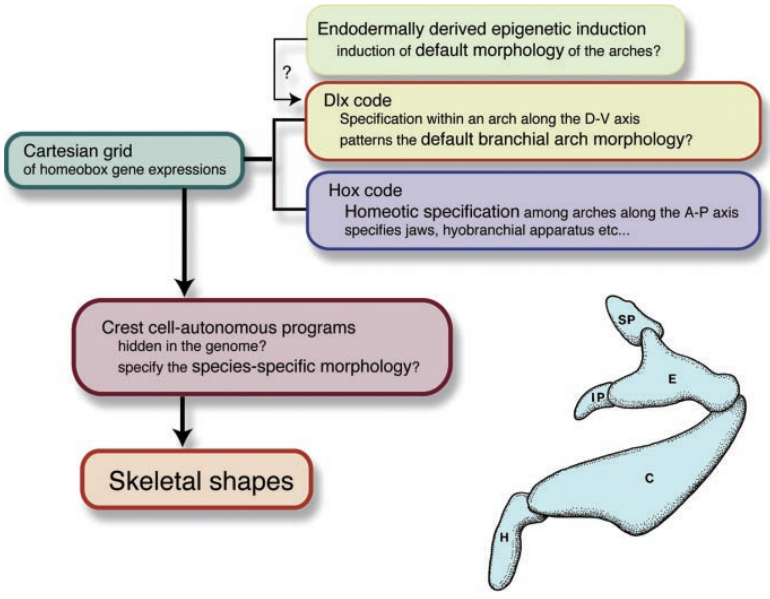


**Fig. 13.** How many shapes? How are they related to each other? The cartesian grid pattern of homeobox gene expression patterns consists of the *Hox* code and *Dlx* code. The *Dlx* code appears to define the dorsoventral specification of a single arch, which is patterned primarily based on the default morphology of the branchial skeleton recognized by comparative morphologists (below). The Hox code, on the other hand, defines the anteroposterior positional values for the arches, defining the identities of the mandibular, hyoid, more posterior arches, and so forth. Therefore, these developmental systems are apparently the factors that allow the continued use of the terminology of comparative morphology for the vertebrate branchial arch skeletons (left below; based on Portmann, 1969). In other words, the developmental mechanism behind this level of skeletal specification and comparative morphological recognition (morphological homologies) are isomorphic to each other. There should be another factor to explain the actual shape of the skeletal elements, which differs in each animal species. This developmental program, as implied by Schneider and Helms (2003), appears to proceed mainly (if not exclusively) within the crest cell lineage. *Abbreviations: C*, ceratobranchial; *E*, epibranchial; *H*, hypobranchial; *IP*, infrapharyngobranchial; *SP*, suprapharyngobranchial.

Footnote:
According to recent work by Trainor *et al.* (2002, 2003), fibroblast growth factor 8 (FGF8) derived from the midbrain-hindbrain boundary of the embryo downregulates *Hox* expression in the rostral hindbrain neural crest or crest cells destined to populate PA1 (Fig. 4C). When the graft was devoid of this FGF8-producing domain, the normal Hox code was restored in the chimeric embryo, and normal cartilage appeared in the hyoid arch, even if it had received ectopic neural crest cells. *Hox* regulation depends on the environmental signals that induce PA crest cells to express the correct set of *Hox* genes (Hunt *et al*., 1998). However, the experiment of Trainor *et al.* (2002) does not necessarily exclude the possibility of neural crest precommitment in skeletogenesis, because the rostral midbrain crest can still form ectopic mandibular arch elements in the second arch of the chimera, without any FGF8 production activity in the graft itself. Moreover, a series of experiments reported by Couly *et al.* (1998) apparently contradicts that of Trainor *et al.* (2002). More analytical experiments are required to clarify the mechanism of *Hox* regulation, and the developmental significance of the Hox-code default in PA1.

entiate (Fig. 9; also see Kuratani *et al*., 1997, 2004, for a reviews). Thus, the prechordal cranium can be viewed not only as the crest-derived rostral half of the neurocranium, but also as the skeletal part differentiated from the rostral half of the trigeminal crest cells, which should be called the 'premandibular' crest cells (Fig. 6).

In certain classical concepts, the term 'premandibular' used to imply the presence of another pharyngeal arch in front of the mandibular arch of the ancestral vertebrates, which is not now generally accepted. The rod-like shape of its skeleton, the trabecular cartilage, resembles the pharyngeal arch. More posterior mesodermal neurocranial elements, the parachordal cartilages, are also paired and rod-like; for trabecula cranii, see de Beer, 1931, 1937; for a review of the cartilage of the same name in the lamprey, see Johnels, 1948; Kuratani *et al*., 2001, 2004). The idea of the 'premandibular arch' was purely idealistic (for the reinterpretation of agnathan fossil evidence, see Janvier, 1996; also see Kuratani, 2005 in press, for trabecular cartilage and premandibular region). It was assigned to the hypothetical head segment to which the ophthalmic nerve and premandibular mesoderm belong, as opposed to the relatively clearer mandibular segment that includes the mandibular arch, the maxillomandibular portion of the trigeminal nerves, and the mandibular head cavity of the shark. Obviously, this schematic interpretation of the vertebrate head is based on the assumption that the hypothetical mesodermal segments (refuted above) and the branchiomeric pharyngeal arches are associated with each other in a one-to-one fashion (reviewed by Kuratani, 2003). If we are to assume such a unified segmental scheme for the vertebrate head in the context of recent evolutionary developmental theory, there should be a single common generative constraint that affects the segmental organization of the pharyngeal arches, mesoderm, and possibly the rhombomeres together, such as a particular upstream developmental event. However, no such developmental event has so far been identified.

Disregarding the segmental organization of the vertebrate head, there is undoubtedly a large ectomesenchymal part in the trigeminal crest cells (see Kuratani, 1997, 2004a; Kuratani *et al*., 2004, for definition), rostral to the PA1 crest cells (Fig. 14). Early in chicken embryogenesis, the trigeminal crest cells form a continuous sheet of cells with no

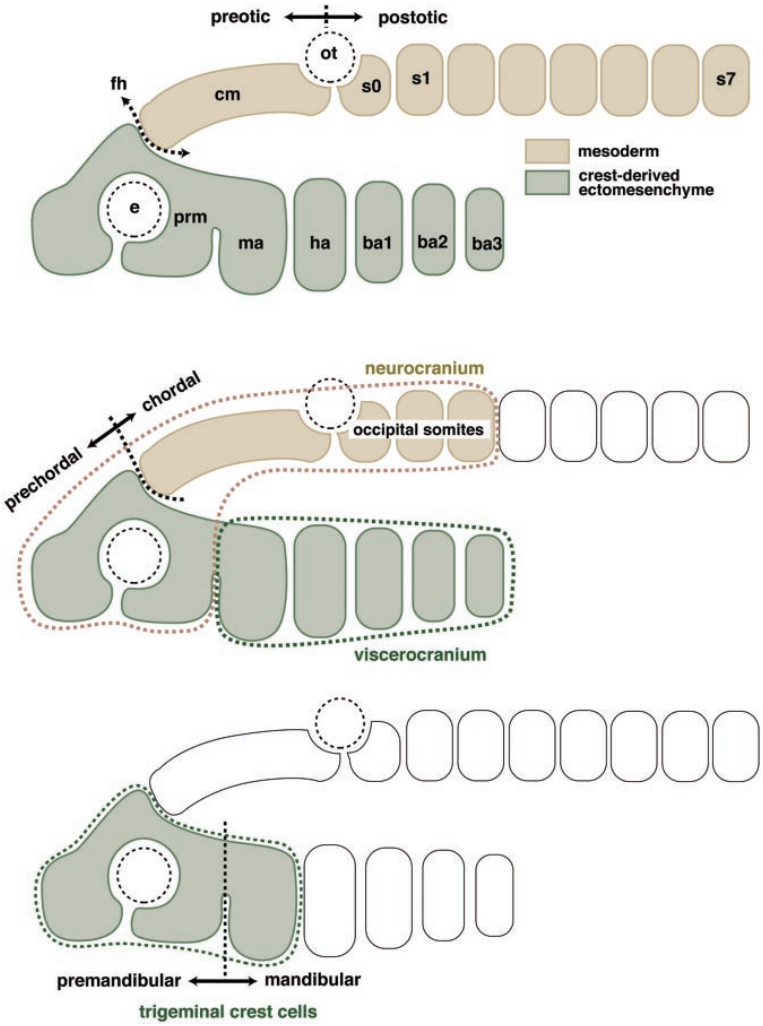


**Fig. 14.** Divisions of the craniofacial mesenchyme. The craniofacial mesenchyme can be named and classified in different ways according to the methods of identification. Top: The mesodermal mesenchyme and crest-derived ectomesenchyme are recognized based on cell lineages. At the level of the otocyst (ot), the mesodermal component is further divided into the preotic region, or the unsegmented cephalic mesoderm (cm), and the truly segmented postotic region, which is divided into somites (s0-s7). In the neurocranial region, the cephalic mesoderm and crest cells form an interface at the level of the hypophysial foramen (fh), or the rostral tip of the notochord (see Fig. 10). Middle: The distinction of the neurocranium and viscerocranium does not precisely coincide with the distinction of cell lineage. The neurocranium consists of the rostral crest-derived part and the caudal mesodermal part. Moreover, the mesodermal neurocranium of many vertebrate species includes the postotic somites as the source of the occipital bone, the only truly segmented portion of the mesodermal neurocranium. Bottom: The most rostral cephalic crest cell population is called the trigeminal crest cells. The posterior part of this cell population is incorporated into the mandibular arch (ma). The rest of the trigeminal ectomesenchyme is now referred to as the 'premandibular' region (prm), corresponding to the 'prechordal' region of the neurocranium of Couly *et al*. (1993) (see the middle scheme). The transcendental idea of the 'premandibular arch' derives from this schema. *Abbreviations: ba1-3*, branchial arches; *e*, eye; *ha*, hyoid arch.

boundary. However, through the inductive action of the FGF8 localized in the ventral ectoderm, the caudal half of the cells are specified as mandibular crest cells, with the rest defined as premandibular crest cells (Shigetani *et al*., 2000; reviewed by Kuratani, 2005 in press). Based on the regionalized deployment of crest cells, as shown by Köntges and Lumsden (1996), the premandibular crest cells appear to originate from the neural crest between the forebrain and rostral midbrain (also see Osumi-Yamashita *et al*., 1994). However, this does not mean that these cells are precommitted to the premandibular structures at the premigratory state. Therefore, the putative premandibular crest-cell-derived skeletal elements, such as the trabecula and nasal capsule, differentiate after the premandibular-mandibular specification of trigeminal crest cells. Moreover, like the rest of the PA skeletons, the premandibular skeletal elements require the presence of endoderm, but not the notochord, to chondrify.

If they lack several developmental and morphological features required for them to be called 'pharyngeal arches', the premandibular and PA ectomesenchyme are similar in the way they chondrify. For example, the premandibular part of the cranium depends on an interaction with the endoderm (Couly *et al*., 2002) not just with the forebrain and ectodermal epithelium (Noden, 1978). As noted above, these skeletal elements are called the 'prechordal' cranium, as opposed to the 'chordal' cranium, in terms of distinct cell lineages and specific signaling mechanisms of skeletogenesis (Figs. 10, 13). Viewed earlier in embryogenesis, when a continuous ectomesenchyme is secondarily regionalized through tissue interactions into the segmented pharyngeal region and the part rostral to it (out of the segmental context), we recognize a distinction between the 'premandibular' (perhaps more suitably called the 'pre-pharyngeal') and the 'mandibular' ectomesenchyme (Fig. 14). Although the former is integrated into the neurocranium in a functional sense, as supposed by comparative embryologists, it simultaneously represents the most rostral component of the crest-derived cranium that depends on the presence of cephalic endoderm (see below). Branchiomeric pharyngeal arch ectomesenchyme, in this context, would be more suitably viewed as a secondarily segmented part of the vertebrate head that is filled by cephalic crest cells (see Fig. 1 of Kuratani, 2005 in press). The presence of crest-derived ectomesenchyme *per se*, does not necessarily predict the presence of branchiomeric segments, nor is it necessary to assume a vertebrate ancestor in which the cephalic ectomesenchyme was completely segmented.

**Cartesian grid of homeobox gene expression and environmental cues**

Like the Hox code that functions along the anteroposterior axis, the *Dlx* genes are expressed in a similar nested pattern in the mouse, and possibly in other gnathostome embryos. In the mouse, *Dlx1* and *Dlx2* are expressed rather ubiquitously in the PA ectomesenchyme, whereas the expression of *Dlx5* and *Dlx6* is restricted to the ventral half of PAs (Fig. 4). Furthermore, *Dlx3* and *Dlx7* are expressed only in the distal (ventral) tips of the PAs, completing the nested pattern of *Dlx* gene expression, referred to as the Dlx code (Depew *et al*., 2002). These genes seem to pattern the arch skeleton along the dorsoventral axis, because the simultaneous disruption of *Dlx5* and *Dlx6*, the genes restricted ventrally, results in a mirror-image duplication of the upper jaw elements in place of the lower jaw elements (also see Beverdam *et al*., 2002; for mutants of other *Dlx* genes, see Qiu *et al*., 1995, 1997; and Ozeki *et al*., 2004, for related phenotypes). Therefore, in terms of developmental programming, it is the upper jaw morphology that is the default identity, and the patterning of the lower jaw may have evolved secondarily downstream from the ventrally expressed transcription factors (Depew *et al*., 2002). It is possible then, that such a *Dlx* code was a prerequisite for the dorsoventral specification of the PA skeleton, including the jaws (reviewed by Schilling, 2003), and the lamprey, with its dorsoventrally symmetrical pharyngeal arch skeleton, may not have arrived at that stage of evolution (Neidert *et al*., 2001; Myojin *et al*., 2001; reviewed by Schilling, 2003, and Shigetani *et al*., 2005 in press).

Interestingly, in both *Dlx5*/*Dlx6* double-knock-out and *Hoxa-2*-disrupted mice, duplicated skeletal elements showed symmetrical patterns with respect to the original skeletal elements (Depew *et al*., 2002). The cartilage of the lamprey pharyngeal arch basket also shows dorsoventral symmetry, and we find the pharyngeal pouches on the axis of this symmetry. This coincidence implies an inductive function of the pharyngeal endoderm in skeletal patterning, which suggests that the Hox and Dlx codes are simply systems that provide positional cues, and do not actually shape the skeleton. It might also explain why the *Hox*-negative crest always produces the same part of the mandibular arch skeleton (jaw articulation) when placed at the level destined to end in the second arch (Couly *et al*., 1998). Recently, such endodermally derived inductive activity was exemplified in the chicken embryo.

Couly *et al.* (2002) removed each part of the rostral endoderm from stage 8 chicken embryos, and showed that a different part of the crest-derived cranial skeleton was lost in each case, depending on the anteroposterior level from which the endoderm was removed. The most rostral endoderm, or the preoral gut, was required for the chondrification of the prechordal (premandibular) cranium, and the slightly more posterior level of the endoderm for the rostral tips of the mandibular arch skeleton, and so forth. Similarly, ectopically implanted endoderm induced skeletal elements with specific identities and orientations, depending on the origin and orientation of the grafted endoderm. These experiments suggest that a schematic representation of the crest-derived skeletal identities can be drawn on the endodermal sheet, which is organized as a lattice defined by the anteroposterior and dorsoventral axes. However, the story is not that simple because; (1) inactivation of the *Hox* gene function in

the second arch still results in the transformation of the hyoid arch skeleton into the identity of the mandibular arch, where normal endodermally derived inductive events still occur; and (2) as shown by Wagner (1959), the species-specific shape of the crest-derived skeleton appears to be coded in the premigratory crest, not in the host environment, including the endoderm. How can we reconcile these apparent discrepancies?

In response to the first point, we can predict that the endodermally derived signaling may be virtually the same for the ectomesenchyme of the mandibular arch and the hyoid arch (but also see Ruhin *et al*., 2003). The *Hox* function would 'modulate' the downstream differentiation process, resulting in the two different identities of the skeleton. The second point, on the other hand, seems to force us to divide the concept of the 'shape' or 'identity' of the skeleton into several different levels or types. The endoderm sends towards the crest cells a signal that defines the morphological identities, such as 'quadrate' or 'articular' that are commonly found in different groups of animals. The crest cells translate these signals using their own genomes to confer the actual shape, which is unique to each animal group. If the contents of the endodermally derived signals are somewhat similar to the framework at the level of comparative morphology, the response of the crest cells would be more like the actual animal shape, which could be more or less cell autonomous in the crest cell lineage. The presence of these different levels of morphogenesis has been alluded to in a unique experiment performed recently by Schneider and Helms (2003).

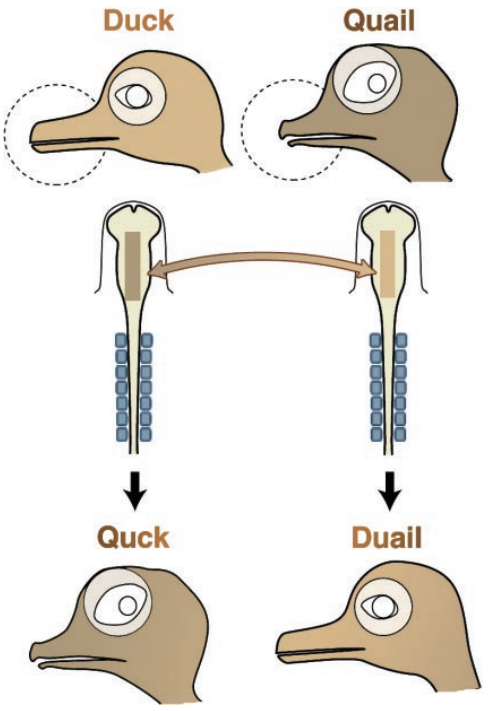


**Fig. 15.** Does species-specific morphology reside in the crest cells? Based on the experiment by Schneider and Helms (2003). When the cephalic neural crest is exchanged between the duck and quail, the morphology of the ectomesenchymal part of the head develops into the identity of the crest donor species. Because the developmental patterning of the crest-derived cells requires the embryonic environment including the endoderm and ectoderm (host tissue), this experiment implies there is a developmental process that generates species-specific traits associated with the crest cell lineage, more or less independent of the epigenetic, tissue interactions.

**What determines the shape? How many types of shapes?**

The cephalic *Hox* code in the pharyngeal arches first appeared to fit the earlier data in the experimental embryology. It had been believed that the skeletal shape is predetermined in the premigratory neural crest. In the context of 'skeletal identity', this precommitment of the neural crest tended to be oversimplified, and positional values and species-specific morphology were often confused. For example, interspecific transplantation of the crest between *Tritrus* and *Bombina* resulted in a skeleton with the donor morphology in the chimera (Wagner, 1959). Noden's experiment (Noden, 1983), on the other hand, was not exactly relevant to the above question, but to the positional value of the pharyngeal arch ectomesenchyme along the anteroposterior axis.

Schneider and Helms (2003) exchanged premigratory cephalic neural crest between duck and quail embryos, bird species with distinct craniofacial morphologies (Fig. 15). Interestingly, the shape generated in the chimera was always more similar to that of the donor species than that of the host. Therefore, as in the experiment of Wagner (1959), who used two amphibian species, the 'shape' of the chimeric skeleton resided in the crest cells. The embryonic environment of the host tissue probably sent the same inductive signals, but the crest cells that received those signals could only respond based on the genome present in their nuclei. The quail crest cells did not know how to assemble as a 'duck quadrate' when they received an order from the duck embryonic environment 'to make the quadrate'.

The experiments of Schneider and Helms (2003), as well as that of Wagner (1959), imply that there can be at least two meanings to the 'shape' of a cartilage: the 'species-specific visible shape' and the 'equivalent identity' of the skeletal elements, as we call two different skeletal elements in two different animals species the same name in comparative morphology (Fig. 13). We must bear in mind that the concept of morphological homology does not require any resemblance of actual shape or function, but should be based on equivalent relative positions in the shared body plan. Again, 'shared topographical position' denotes identical epigenetic induction in both tissues. If such an interaction is evolutionarily fixed and unchangeable, this immutability will be recognized as a developmental constraint that generates the 'morphological homology'. This is close to the idea that the phylotypic stage of animal development tends to be conserved through a complicated net-

work of global interactions (Sander, 1983; Elinson, 1987; Raff, 1996), and that the embryonic patterns found at the phylotypic stages are the source of most global homologies that define the body plans of animal phyla.

Importantly, these different levels of concepts can be clarified by appropriately designed experiments and a precise understanding of the developmental patterning mechanisms. As an analogy, the idea of 'transposition' proposed by Goodrich (1910, 1930) to explain the variable vertebral formulae, and 'transformation and metamerism' proposed by Goethe (1790) who established the *Morphologie* itself, and the concepts of 'meristic' and 'homeotic' mutations proposed by Bateson (1894), clearly predicted the nature of morphogenetic system dependent upon the *Hox* code.

**Conclusions and perspectives**

The experiment of Noden (1983) involving transplantation of the mandibular crest to the level of the hyoid, and that of Trainor *et al.* (2002), which implies an epigenetic function of midbrain-hindbrain boundary must be reconciled with that of Couly *et al.* (1998) in the context of regulation of the cephalic *Hox* code and its maintenance (or restoration). Undoubtedly, there is a certain level of environmentally derived signals that maintains or upregulates the *Hox* gene expression, as predicted by Hunt *et al.* (1998), who rotated the whole hindbrain along the anteroposterior axis and had restored the correct Hox code. Simultaneously, when grafted crest cells formed a large cell population, there would have been a community effect that would maintain the same original *Hox* gene expression under a varied environment, leading us to believe that *Hox* regulation in the crest is, at least as a phenomenon, precommitted at the premigratory state along the neuraxis.

Importantly, the segmental deployment of crest cells and the expression of *Dlx* and *Hox* genes are spatiotemporally highly organized at the stage of phylotype, on which both the developmental specification and evolutionary changes are dependent. No doubt the acquisition together of such an organized embryonic pattern and gene expression patterns is one of the most crucial factors in the morphogenetic events of the vertebrate cranium. It is highly conceivable that such patterns were necessarily stabilized through evolutionary selection; the developmental mechanism and genes could change without altering the patterns generated. Furthermore, the pseudosegmental patterns in the vertebrate phylotypic cranium may be the most important developmental factor (developmental constraint) in the morphological homology of skeletal elements. This pattern is obtained secondarily in embryonic development, and is not present in very early embryos. In this context, it is worth mentioning that the results of mapping studies performed in the cephalic mesoderm of two different stages of chick embryos by Couly *et al.* (1992) and Noden (1988) differ greatly (Fig. 6). The fate map at the late neurula is reminiscent of Goodrich's segmental theory, whereas such a pattern is not yet established when the fate mapping is performed at earlier stages.

In conclusion, comparative embryology of the vertebrate cranium has shown the presence of a developmentally constrained pattern of embryos, and the resulting tissue interactions that give rise to certain specific patterns of skeletal elements. We can now identify the types of interactions and cell movements that are crucial in the generation of certain specific morphological patterns, and the developmental and evolutionary contexts that must be addressed to better understand craniogenesis. With the molecular developmental and genetic techniques available to us, the longstanding question of the 'vertebrate head' has now reached its final stage of resolution.

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