REVIEW ARTICLE

History of Studies on Mammalian Middle Ear Evolution: A Comparative Morphological and Developmental Biology Perspective

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ABSTRACT

The mammalian middle ear represents one of the most fundamental morphological features that define this class of vertebrates. Its skeletal pattern differs conspicuously from those of other amniotes and has attracted the attention of comparative zoologists for about 200 years. To reconcile this morphological inconsistency, early comparative morphologists suggested that the mammalian middle ear was derived from elements of the jaw joint of nonmammalian amniotes. Fossils of mammalian ancestors also implied a transition in skeletal morphology that resulted in the mammalian state. During the latter half of the 20th century, developmental mechanisms controlling the formation of the jaw skeleton became the subject of studies in developmental biology and molecular genetics. Mammalian middle ear evolution can now be interpreted as a series of changes in the developmental program of the pharyngeal arches. In this review, we summarize the history of middle ear research, highlight some of the remaining problems, and suggest possible future directions. We propose that to understand mammalian middle ear evolution, it is essential to identify the critical developmental events underlying the particular mammalian anatomy and to describe the evolutionary sequence of changes in developmental and molecular terms. We also discuss the degree of consistency between the developmental explanation of the mammalian middle ear based on molecular biology and morphological changes in the fossil record. *J. Exp. Zool. (Mol. Dev. Evol.) 314B:417–433, 2010.* © 2010 Wiley-Liss, Inc.

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Unlike nonmammalian amniotes, which have only one ossicle, the columella auris (Fig. 1A, C, E), the mammalian middle ear has three ossicles, the malleus, incus, and stapes (Fig. 1B, D, F). The evolutionary origins of this complex and its homology have been regarded as among the most formidable conundrums in vertebrate comparative morphology. Why is a difference in the number of the ossicles a problem? It is problematic because animals are usually unable to generate entirely new anatomical elements de novo in evolution. Geoffroy Saint-Hilaire, a French anatomist, pointed out in 1818 that equivalent sets of skeletal elements are connected in an identical order in all animals, and proposed that every animal skeletal type was derived from changes to a common skeletal pattern. This *principe des connexions* remains one of the simplest definitions for morphological homology (reviewed by Hall, '98). According to this law, two of the three ossicles in the mammalian middle ear must have their homologues in the nonmammalian skull, a phenomenon that has puzzled morphologists for many years.

From the perspective of comparative morphology, the vertebrate head exhibits a series of equivalent modules called the pharyngeal arches (Fig. 1G). The first, the mandibular arch,
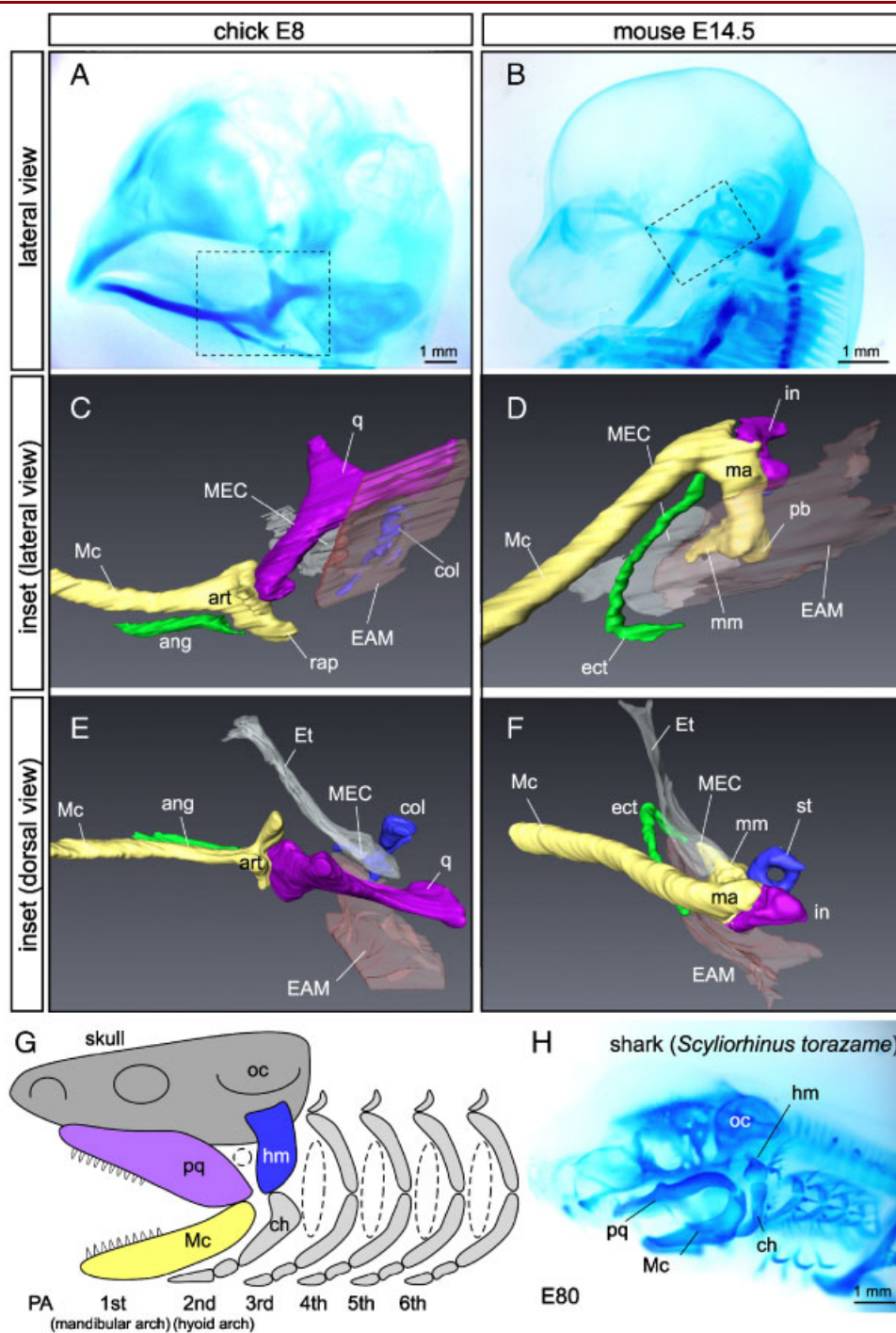
Figure 1. (A–F) Morphological structures in chick embryonic day (E) 8 (A, C, E) and mouse E14.5 (B, D, F) embryos. (A, B) Cartilage elements stained with alcian blue. (C–F) Three-dimensional reconstructions of the insets in A and B. Lateral (C, D) and dorsal (E, F) views of skeletons with a developing tympanic membrane. (G) Lateral view of the skeletal structure of an idealized gnathostome head. The pharyngeal arches show repeated structures. The hatched lines indicate pharyngeal slits, which were originally gill holes. (H) Cartilage elements of a shark (*Scyliorhinus torazame*) E80 embryo stained with alcian blue. See the list of abbreviations for the anatomical nomenclature used in this and the following figures.

comprises the upper and lower jaws, called the palatoquadrate and Meckel's cartilage, respectively (Fig. 1G). The second, the hyoid arch, is also subdivided dorsoventrally. The dorsal moiety is called the hyomandibular and the ventral moiety the ceratohyal (Fig. 1G), although an intercalated element between the two elements, such as stylohyal, is often seen in many vertebrates (Goodrich, '30). The simplest configuration of the latter arch is evident in elasmobranchs (Fig. 1H). In comparative morphology, all the visceral arches are regarded as serial homologues, and Geoffroy Saint-Hilaire first drew the conclusion that the mammalian middle ear ossicles are homologous with the opercular bones in teleosts (Geoffroy Saint-Hilaire, 1818; reviewed by Appel, '87).

Subsequently, several famous morphologists such as Meckel (1820), Huschke (1824), Rathke (1832), and Burdach (1837) addressed this issue, but only Reichert (1837) was able to formulate a hypothesis that survives today. He dissected pig embryos with needles under a microscope and realized that two of the mammalian ossicles, the malleus and incus, were derived from cartilages equivalent to the lower and upper jaw elements in other amniotes: the articular and quadrate. Modern histological observations have also shown that the incus arises from the posterior part of the palatoquadrate as a primordium connected to the basal part of the ala temporalis (the ascending process of the palatoquadrate) by a thread of connective tissue (Presley and Steel, '76), which confirms Reichert's interpretation. The connective tissue thread often chondrifies to form a cartilaginous element of the first arch domain when one of the genes expressed in the first arch ectomesenchyme is knocked out (reviewed by Smith and Schneider, '98). Thus, the connection between the incus and the rest of the upper jaw has been elucidated.

Geoffroy's concept of homology was also applied to muscles and nerves, and a consistent branchiomeric scheme in the mammalian middle ear was confirmed: m. tensor tympani and m. tensor veli palatini are derivatives of the first arch and are innervated by the trigeminal nerve that innervates first arch derivatives exclusively. Similarly, m. stapedius is a second arch derivative innervated by the facial nerve (Rabl, 1887). The German comparative morphologist Gaupp ('11a,b, '12) examined a huge number of skulls of various animals and published a 400-page monograph titled "Die Reichert Theorie" (Gaupp, '12). He pointed out that, unlike the nonmammalian gnathostome jaw, in which the "primary jaw joint" consists of the quadrate and the articular, mammals have a unique "secondary jaw joint" between two dermal elements, squamosal and dentary (Gaupp, '11a,b, '12). Gaupp's morphological insight influenced subsequent evolutionary studies on the middle ear, based on the fossil record and embryology (see below).

Reichert (1837) suggested that the stapes is homologous to the columella auris in nonmammalians as an element homologous to the hyomandibular (Fig. 1C–G). As the embryonic second arch arises at the level of the inner ear, the dorsal end of the

hyomandibular must abut the otic capsule of the neurocranium (Fig. 1G). This topographical relationship seems to have facilitated the subsequent evolution of the hyomandibular. As a device that anchors the upper jaw, this skeletal element is often present in primitive gnathostomes, including extant elasmo-branchs (Fig. 1H). It thus connects the quadrate (or jaw joint) and the neurocranium. In this type of jaw suspension, called a hyostyly, the upper jaw is fixed to the neurocranium via the hyomandibular (Huxley, 1876; reviewed by Goodrich, '30). Thus, the mammalian middle ear exhibits a pattern topographically identical to that of the hyostylic skull. By contrast, in most extant tetrapods the quadrate is attached firmly to the cranium to establish an autostylic jaw suspension that does not involve the hyomandibular (Huxley, 1876). The latter suspension was thought to have evolved in primitive tetrapods, in which the hyomandibular assumed a hearing function (Goodrich, '09, '30; de Beer, '37; but see also Clack, '89, '93, 2002a,b; Clack et al., 2003; Brazeau and Ahlberg, 2006). Thus, mammalian middle ear evolution was tightly coupled with the evolution of jaw suspension (reviewed by Clack, 2002a).

FOSSIL EVIDENCE

Since the 1840s, fossil records from South Africa and Russia have provided further evidence to support the primary jaw joint origin of the mammalian middle ear, in a pattern consistent with Reichert's, Rabl's, and Gaupp's theories. These fossils are synapsids, a class of animals that includes the pelycosaurs and therapsids, from which mammals emerged (Fig. 2A). Studies of these mammal-like reptiles showed that the anatomical patterns of the middle ear have gradually transformed the elements from the primary jaw joint to the mammalian middle ear components (Owen, 1845; Broom, '11; Gregory, '13; Westoll, '43, '44, '45; Olson, '44; Parrington, '55; Hopson, '66; Allin, '75; Crompton and Jenkins, '79; Lombard and Bolt, '79; Crompton and Sun, '85). In the pelycosaurs (sphenacodontids, such as *Dimetrodon*), the postdentary elements (articular, surangular, and angular) and the quadrate were large (Fig. 2A). In early therapsids (theroce-phalians), there was a slightly advanced state in which the postdentary elements became smaller. These were incorporated into the auditory organ of primitive mammals and the dentary expanded posteriorly as well as dorsally to establish the secondary joint with another dermal element, the squamosal (Fig. 2A). Although the angular did not show any conspicuous morphological change in the most primitive pelycosaurs, such as *Ophiacodon*, it developed a posteriorly oriented process in more advanced pelycosaurians, such as *Dimetrodon*, to be called the reflected lamina, which would eventually form the ring-shaped ectotympanic of early mammals (Fig. 2A).

Interestingly, several advanced cynodonts of the late ther-apsidian group seemed to have two functional jaw joints. *Diarthrognathus* possessed a medial endochondral primary jaw joint and a lateral dermally ossified secondary jaw joint
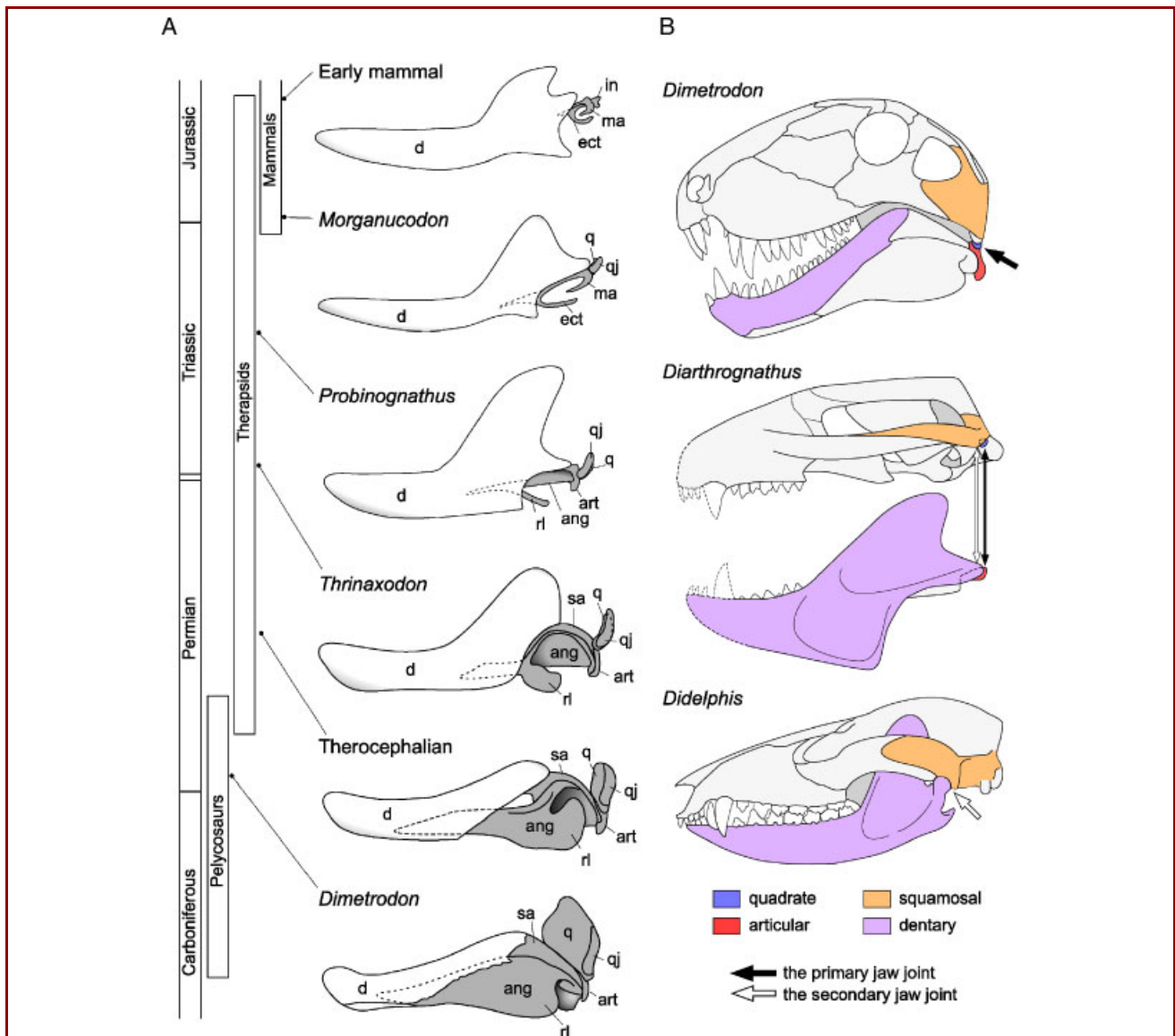
Figure 2. Paleontological evidence for mammalian middle ear evolution. (**A**) Diagrams of lateral views of jaw skeletal elements showing modifications leading to the mammalian condition (after Allin, '75). The geological record and occurrence of each animal are indicated on the left. For clarity of comparison, no teeth are shown. Note that a set of postdentary elements (articular, surangular, and angular) and the upper jaw elements (quadrate and quadratojugal), indicated by gray, became separated from the dentary and reduced in size during the transition from pelycosaurs to mammals. The sequence of changes in the fossil record does not represent a true ancestor–descendent relationship, but only structural grades. (**B**) Changes in jaw articulation during mammalian evolution. In a pelycosaur, *Dimetrodon* (top), the quadrate and articular formed a functional jaw joint (black arrow). In an "advanced" cynodont, *Diarthrognathus* (middle), an additional jaw joint was observed between the squamosal and dentary (white arrow). In an extant marsupial, *Didelphis* (bottom), the functional jaw joint has been taken over only by the squamosal and dentary.

(Crompton, '63, '72; Fig. 2B). This condition is highly suggestive of a transitional state in mammalian evolution and implies that, starting from a double-joint state, the laterally situated dermal secondary jaw joint came to serve for feeding, releasing the medially located primary jaw joint elements from their original function, permitting them to move medially into the middle ear. A developmental phenomenon also supports the above scenario. Thus, in marsupial embryos, the angular shows a conspicuous

similarity to that of a cynodont before it takes the typical shape of the ectotympanic (Palmer, '13). Interestingly, the offspring of marsupials initially use the primary jaw joint to suck the mother's nipples in the pouch, because their squamosal and dentary bones are too premature to form a functional joint at this stage of development (Crompton and Parker, '78; Maier, '87a; but see Filan, '91). Marsupials are thus in a sense born as "reptiles" before they become true mammals in the mother's pouch. However, it remains to be determined whether the skeletal pattern of marsupial pouch young truly recapitulates the ancestral condition or whether it is merely a secondary adaptation prompted by a need for early suckling. In this context, it should be noted that the mammalian developmental sequence does not simply recapitulate the *Diarthrognathus*-like condition, in which double joints are arranged mediolaterally. Instead, the primary and secondary joints seem anteroposteriorly, as has been pointed out earlier (see, for example, Fuchs, '05, '31; Jarvik, '80; Fig. 2B). The driving force for the shift from the primary to secondary jaw joints has also been controversial. One explanation would be that the increase in masticatory capabilities associated with expansion of the dentary was impelled by selective forces, and only when postdentary elements became vestigial did they assume an auditory role and enter the middle ear (Parrington, '79). Another possibility is that the postdentary elements served as middle ear components in the early mammalian ancestors and auditory adaptation was an important factor in this sequence of morphological evolution (Allin, '75).

The earliest forms generally classed as mammals (mammaliaformes; Rowe, '88), such as *Morganucodon*, seemed to have utilized only the secondary jaw joint, but the postdentary elements were still fused to the jaw skeleton (Kermack et al., '73; Crompton and Luo, '93; Fig. 3A). These elements seem to have been detached from the dentary and completely incorporated into the middle ear, which is referred to as the definitive mammalian middle ear (DMME). Precisely how and how many times the event occurred is controversial. Although it was suggested that brain expansion increased the distance between the postdentary elements and dentary to separate them during mammalian evolution (Rowe, '96a,b; Luo et al., 2001), this view was refuted by a fossil with a small brain size and with postdentary elements separate from the dentary (Wang et al., 2001). An early Cretaceous eutriconodont mammal, *Yanoconodon*, was recently found in northeastern China (Luo et al., 2007). In this animal, the malleus, incus, and ectotympanic were almost completely preserved at the posterior tip of the ossified and twisted Meckel's cartilage (Fig. 3B). It is noteworthy that the morphology of the postdentary elements shows significant similarities to that of the extant platypus (Fig. 3C, D). In extant mammals, Meckel's cartilage disappears during development and the middle ear loses its connection with the lower jaw (Fig. 3C, D). On the other hand, in ancestral mammals, Meckel's cartilage seems to have ossified and thus the postdentary elements
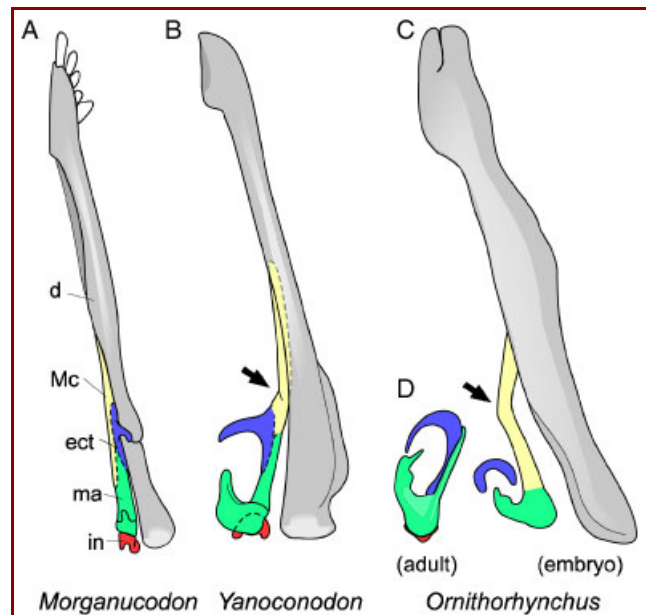

Figure 3. Evolution of the lower jaw skeleton in ancestral mammals (based on Luo et al., 2007). (**A**) Ventral view of the lower jaw of the most "primitive" mammal, *Morganucodon*. The ectotympanic and malleus are completely in contact with the dentary. By contrast, in *Yanoconodon* (**B**), the ectotympanic and malleus are connected anteriorly to the dentary via an ossified Meckel's cartilage, but these are mediolaterally separated from the posterior part of the dentary, facilitated by curvature of the cartilage (arrow). A similar condition is seen in an extant monotreme embryo, *Ornithorhynchus* (**C**). The middle ear bones of an adult *Ornithorhynchus* (**D**) demonstrate significant similarity to those of *Yanoconodon*. *Yanoconodon* seems to retain the embryonic pattern of *Ornithorhynchus* because of an earlier ossification of Meckel's cartilage, but otherwise its ectotympanic, malleus, and incus are almost identical to those of the adult *Ornithorhynchus*.

maintained their connection with the lower jaw. The evidence from *Yanoconodon* suggests that there were two evolutionary steps in the establishment of the DMME: first, a mediolateral separation of the malleus and incus from the dentary, and second, a loss of the anterior connection to the dentary resulting from absorption of Meckel's cartilage in later stages. *Yanoconodon* thus seems to be the missing link in the intermediate evolutionary stage of mammals. Although the single vs. multiple origin of the DMME is still controversial (Allin and Hopson, '92; Luo et al., 2007), a *Yanoconodon*-like middle ear was recently found in a more advanced mammal, shedding light on this issue (Ji et al., 2009). Comparative embryological analyses of the middle ear region between monotremes, marsupials, and eutherian mammals have also provided insights into morphological evolution in the mammalian head region (Maier, '87b; Presley, '93; Zeller, '93).

WHERE IS THE BOUNDARY BETWEEN THE FIRST AND SECOND ARCHES?

Although the fossil record has supported Reichert's theory, several theories opposing his theory were put forward based on detailed embryological analyses. The Reichert theory postulated that the malleus and incus are derivatives of the first arch (Fig. 1G), but the boundary between the first and second arches in the skeletal elements of the middle ear was controversial. In the 1960s, Hanson and his co-workers at the University of Chicago reexamined the embryonic development of the mammalian middle ear and posited a dynamic developmental change in the interface between the mesenchymal cells of the first and second arches: a bridge was observed between the procartilaginous anlagen of the two arches. Based on these observations, an inclusion of second arch mesenchyme was suggested to occur during formation of the ventral halves of the malleus and incus, namely the malleus manubrium and the crus longum, the long limb of the incus (Anson et al., '60; Hanson and Anson, '62; Strickland et al., '62; Fig. 4A). Based on congenital abnormalities in humans and the hypotheses put forth by Fuchs ('05, '31), who was one of Gaupp's opponents, Otto ('84) suggested that both the malleus and the incus are derived from the second arch (Fig. 4B). On the other hand, Jarvik, a Swedish paleontologist, compared the pharyngeal skeleton of a tetrapod stem group, *Eusthenopteron*, with that of mammals and concluded that fusion of Meckel's cartilage and the malleus occurred secondarily in marsupials, and that the malleus, incus, and stapes are all derived from the second arch (Jarvik, '80; Fig. 4C). Although the claim of the Chicago group was broadly accepted and cited in Gray's Anatomy until recently (Williams, '95), technological breakthroughs in the 1970s, namely, experimental developmental biology studies involving avian embryos and molecular genetic studies involving flies and mice, were needed to provide further insights into the Reichert theory.

MOLECULAR BASIS OF MORPHOLOGICAL IDENTITIES IN PHARYNGEAL ARCHES

Vertebrates are unique in that ectodermally derived neural crest cells (NCCs) form the ectomesenchyme, the source of the craniofacial skeleton. In the past, a simple explanation for the differentiation of middle ear ossicles was that of anteroposterior specification of the cephalic neural crest along the neuraxis, resulting in position-specific morphologies of visceral arch skeletons. For example, Noden ('83) performed a transplantation of the premigratory (epithelial state) neural crest in avian embryos. The neural crest at the posterior midbrain to anterior hindbrain level, which gives rise to NCCs that populate the first arch and differentiate into the upper and lower jaws as well as accompanying bones, such as the squamosal and pterygoid, was transplanted into the middle hindbrain at levels designed to enable the second arch to differentiate into the middle ear ossicle, the columella auris. This chimeric embryo possessed duplicated

first arch elements instead of the second arch derivatives at the level of the second arch, including the columella (Fig. 5B). Thus, the cephalic neural crest seems to be specified anteroposteriorly in terms of the pharyngeal arch identities.

The pharyngeal arch ectomesenchyme originates from the neural crest area ranging from the midbrain to the hindbrain (reviewed by Le Douarin, '82; Noden, '88). Of these, the hindbrain is segmented into rhombomeres (neuromeres in the rhombencephalon; Fig. 5A) and this segmental pattern is parallel to that of the pharyngeal arches. NCCs arising in this region (as well as from the midbrain) are separated into three main streams that migrate into the mandibular, hyoid, and branchial arches located posterior to the inner ear (Kuratani, '97; Kuratani et al., 2001; Fig. 5A). The positional specification of the arches as reported by Noden ('83) is mediated by a collinear nested pattern of Hox genes; the genes toward the 3′ end of the cluster tend to be expressed more anteriorly along the anteroposterior embryonic neuraxis (McGinnis and Krumlauf, '92; Krumlauf, '93; Prince and Lumsden, '94; Mark et al., '95; Fig. 5A). This pattern of Hox gene expression is called the "Hox code." The Hox code in the caudal head region involves the first five paralogues (Hox-1–5) and is also present in the pharyngeal ectomesenchyme and epithelia in a pattern parallel to that in the hindbrain (Hunt et al., '91; Kuratani and Wall, '92; Prince and Lumsden, '94; Fig. 5A). No Hox genes are expressed in the first arch (Fig. 5A). This "Hox default" state is believed to provide the morphological identity of the first arch. This Hox gene expression pattern is conserved among various vertebrates (Frasch et al., '95; Morrison et al., '95; Takio et al., 2004) and each cognate is linked with a specific segmental identity. The influx of NCCs into pharyngeal arches is, thus, the principal basis of this coincidence in segmental registration between the rhombomeres and the pharyngeal arch mesenchyme in vertebrates (Couly et al., '96; Köntges and Lumsden, '96; Fig. 5A) and provides a molecular basis for the specification of arches first realized by comparative morphologists, such as Rabl and Gaupp.

An experiment analogous to that of Noden ('83) was performed 10 years later at the molecular genetics level. When the mouse gene *Hoxa-2*, which seems to specify the second arch (Fig. 5A), was knocked out, the second arch skeletons, such as the stapes and styloid process, were replaced by the second set of first arch elements—demonstrating homeotic transformation of the second arch into the identity of the first (Gendron-Maguire et al., '93; Rijli et al., '93; Fig. 5B). The duplication also occurred in the alisphenoid and pterygoid, showing their first arch origins, as was implied earlier by comparative morphology (Goodrich, '30; de Beer, '37). In addition, ectopic expression of *Hoxa-2* in the first arch resulted in the arrest of the first arch components and partially duplicated second arch elements in mouse and frog embryos (Grammatopoulos et al., 2000; Pasqualetti et al., 2000). Recent knockdown and gain-of-function approaches with zebrafish have yielded second-to-first arch or first-to-second arch
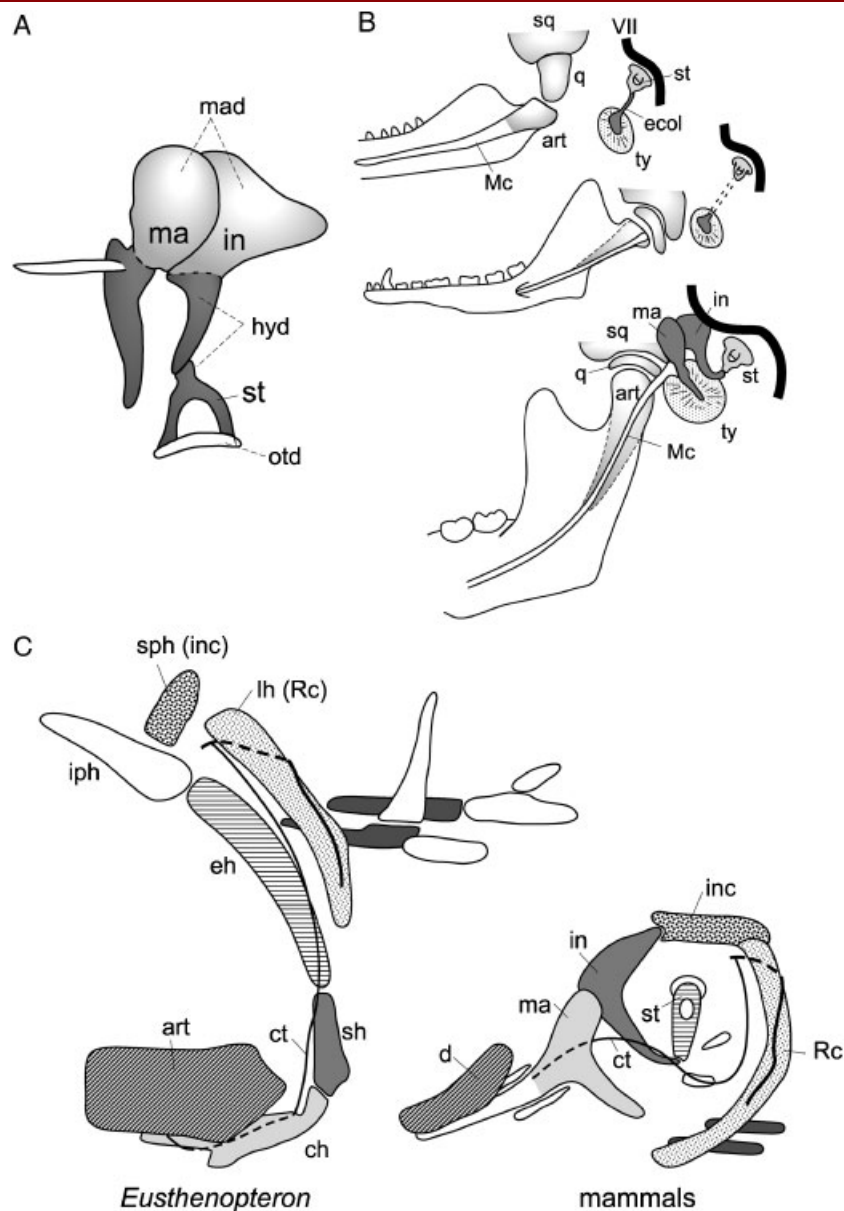
Figure 4. Theories opposed to Reichert's (1837) theory. (**A**) The first and second arch contributions to the middle ear ossicles based on Hanson and Anson ('62). Key: mad, mandibular arch derivative; hyd, hyoid arch derivative; otd, otic capsule derivative. (**B**) The origin of the mammalian middle ear according to Otto's theory ('84). Medial views of reptilian (top), therapsidian (middle), and human (bottom) middle ears. Otto emphasized that human congenital abnormalities implied that jaw and middle ear development are not closely related to each other. He also emphasized Fuchs's theory ('05,'31) that the reptilian jaw joint elements are equivalent to the secondary cartilage between the squamosal and dentary in mammals. Based on these theories, Otto assumed that the malleus and incus were derived from the extracolumella (ecol; dark gray). (**C**) Homology of skeletal elements derived from the second arch between *Eusthenopteron* (left) and mammals (right) according to Jarvik ('80). Each presumed homologous bone is indicated by identical shading. Jarvik assumed that all the mammalian middle ear ossicles are derived from the second arch.
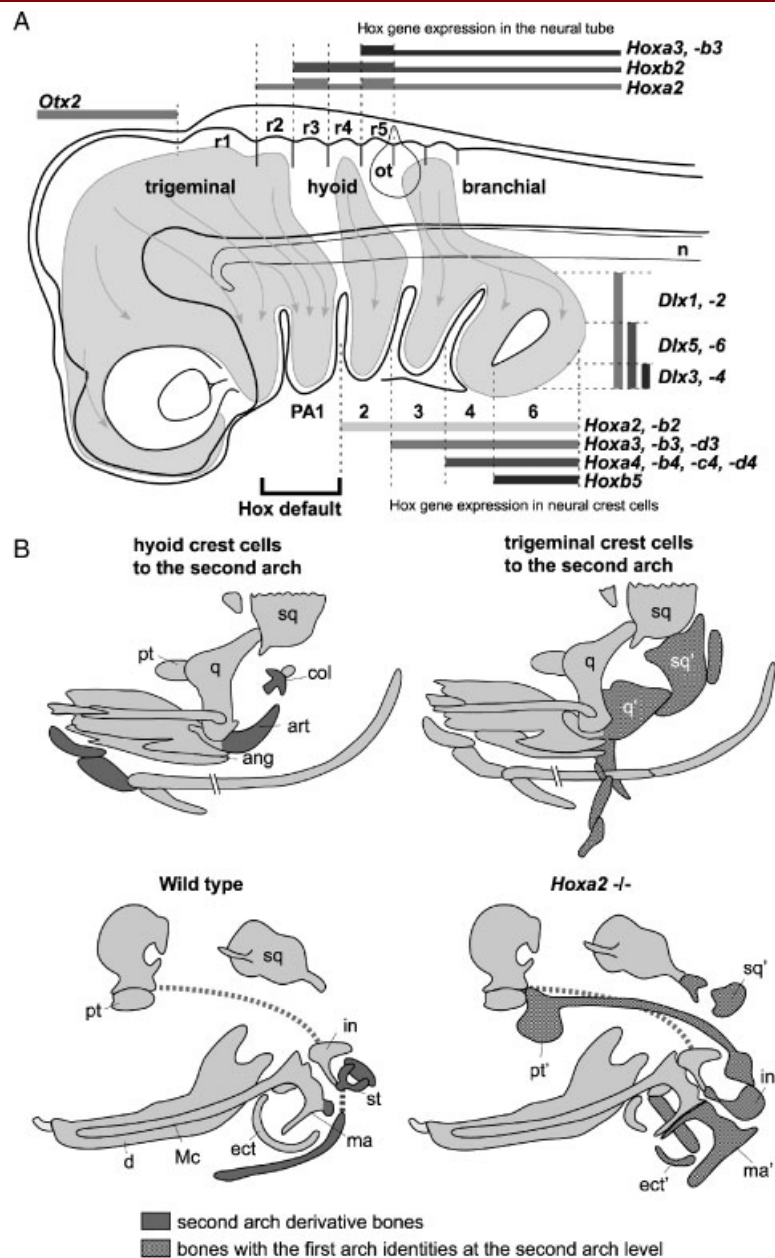
Figure 5. (A) The migration of neural crest cells (NCCs) and Hox and Dlx gene expression patterns in the vertebrate head. The trigeminal, hyoid, and branchial streams of NCCs (gray) migrate into the pharyngeal arches. Hox genes are expressed in nested collinear patterns in the hindbrain and in the NCCs in the pharyngeal arches, constituting positional values for the arches to differentiate into the appropriate morphology. Similarly, Dlx genes are expressed in nested patterns in NCCs in the pharyngeal arches. *Otx2* is expressed in NCCs derived rostral to the boundary between the midbrain and hindbrain. Note that the first arch is specified as a Hox default state. Hox gene expression patterns in the pharyngeal endoderm and ectoderm are not shown in this diagram. (B) Developmental specification of the pharyngeal arches suggested by chicken tissue transplant (Noden, '83) and mouse gene knockout experiments (Gendron-Maguire et al., '93; Rijli et al., '93). Top: When chicken hyoid crest cells were replaced with trigeminal crest cells (right), bones with the morphological identity of the first arch developed in the position that the second arch derivatives occupied in the control embryo (left). Bottom: Similarly, the *Hoxa-2* knockout mouse (right) showed that duplicated first arch derivatives were seen in the position that the second arch derivatives occupied in the control embryo (left). An anatomical name with an apostrophe indicates a duplicated element. Key: r1–r5, rhombomeres 1–5.

homeotic transformations, respectively, suggesting a conserved role for *Hoxa-2* among vertebrates (Hunter and Prince, 2002). Therefore, the molecular genetic evidence strongly supports the serial homology schematic of the visceral arches advocated by the Reichert–Rabl–Gaupp theories.

It is noteworthy that chicken–quail chimera analyses have shown that the retroarticular process, attached to the posterior side of the articular (Fig. 1C), is derived from the NCCs of the second arch (Noden, '83; Köntges and Lumsden, '96; Fig. 5B): a point overlooked in comparative embryology studies (e.g., Goodrich, '30). A recent cell lineage tracing analysis of mouse second arch NCCs showed that the processus brevis of the malleus (Fig. 1D) arises from the second arch (O'Gorman, 2005), which is consistent with evidence that this element disappears after *Hoxa-2* disruption (Fig. 5B). Although the homology of the retroarticular process is controversial (reviewed by Novacek, '93), these results seem to suggest that this structure is homologous with the processus brevis of the malleus. These analyses also revealed that the boundary between the first and second arch should be on the articular. This would explain the inconsistent position of the boundary between the two arches as conceived by the University of Chicago group, which claims that the ventral half of the malleus is derived partially from the second arch (Fig. 4A).

Similar to the role of the Hox code in the anteroposterior specification of the arches, Dlx genes, another class of homeobox gene, are considered to play a role in dorsoventral specification. The nonteleost gnathostome genome possesses six Dlx genes (*Dlx1–6*; Stock, 2005), which also exhibit dorsoventrally nested expression patterns. Thus, *Dlx1* and *Dlx2* are ubiquitously expressed in the pharyngeal arch ectomesenchyme; expression of *Dlx5* and *Dlx6* is restricted to the ventral half, and expression of *Dlx3* and *Dlx4* occurs only in the ventral tips of the arches (Qiu et al., '97; Depew et al., 2002; Fig. 5A). Thus, the Hox and Dlx genes together seem to specify each part of the ectomesenchyme via their Cartesian grid-like expression (Fig. 5A). The function of the "Dlx code" has been demonstrated by loss- and gain-of-function experiments. A double knockout of *Dlx5* and *Dlx6* resulted in transformation of the lower jaw into the identity of the upper jaw and the mutant mouse exhibited mirror-image duplication of the upper jaws (Depew et al., 2002). Equivalent transformations were also observed with knockouts of *Endothelin1* (*Edn1*) or its cognate type-A receptor, *Ednra* (Ozeki et al., 2004; Ruest et al., 2004). *Edn1* is expressed in the epithelia and mesodermal core of the lower jaw region in the first arch, whereas *Ednra* is broadly expressed in the NCCs of the head (Kurihara et al., '94, '95; Clouthier et al., '98). Edn/Ednra signaling was found to activate several genes required for lower jaw specification, including *Dlx6* (Clouthier et al., '98, 2000; Kurihara et al., '99; Charite et al., 2001). Accordingly, ectopic *Edn1* induction in the upper jaw region resulted in transformation of the upper jaw into the identity of the lower jaw, suggesting that the NCCs that migrate into the first arch can form both upper and lower jaw structures and that Edn/Ednra signaling determines which morphogenetic program is activated (Sato et al., 2008).

SIGNALS TO INSTRUCT THE "SHAPE" OF THE SKELETON

How can the NCCs form specific skeletal elements that differ between mammals and nonmammalian amniotes? After Noden's 1983 experiment (Fig. 5A), several subsequent reports suggested that developmental specification of the skeletal shape is more complicated than the idea of predetermined premigratory NCCs, although the idea of a Hox code default state of the mandibular arch (or of the ectomesenchyme therein) is still valid as a prerequisite for jaw specification. For example, any portion of the neural crest between the middle midbrain and r3 levels (the crest destined for the first arch; Fig. 5A) can always duplicate the proximal first arch, including the jaw joint when grafted onto the hyoid arch level (r4) (Couly et al., '98). Also, the Hox code in the pharyngeal arches has been shown to be restored after surgical rotation of the hindbrain neurectoderm along the anteroposterior axis, suggesting that the Hox code is partly regulated and maintained by environmentally derived signals (Hunt et al., '98). In the same context, the Hox code default state in the first arch seems to depend on the midbrain–hindbrain boundary-derived protein FGF8 (Trainor et al., 2002, 2003). Furthermore, local interactions between ectomesenchyme and head endoderm are responsible for morphological specification of the craniofacial skeletons (Couly et al., 2002; Ruhin et al., 2003). Thus, when the rostralmost chicken endoderm was removed from an early neurula, the nasal cartilage disappeared completely. When a slightly more posterior piece of the endoderm was removed, the main part of the lower jaw cartilage was lost. By contrast, when an endoderm graft was transplanted into a normal chicken embryo, skeletal components, corresponding to the endoderm, developed ectopically in the correct shapes, sizes, and directions. Thus, the anatomical patterns of the first arch (and premandibular) skeletal elements are mapped on the rostral endoderm in contact with the NCCs specified by the Hox-code default state.

These endoderm transplantation experiments apparently contradict one of the classical transplantation experiments. Wagner ('59) grafted the premigratory cephalic neural crest between newt and frog embryos and found that the chimera always developed the craniofacial morphology of the crest donor. This apparent contradiction was recently reconciled by Schneider and Helms (2003), who used ducks and quails and showed that species-specific morphological traits primarily reside in a genetic program carried by the crest cell lineage. Thus, we may assume that the evolution of the "species-specific shape" of visceral skeletons is imprinted preferentially onto the developmental program exerted by the ectomesenchyme. Given the putative role of the cephalic endoderm and NCCs in patterning the skeleton, we might have to class the concept of "shape" into different levels: namely, the default morphology of the gnathostome visceral arch

skeleton, the comparative morphological identities (shape as a morphological homology) of each arch or skeletal subset within the arch, and animal species-specific shapes (reviewed by Kuratani, 2005). Thus, although a coherent description of mechanisms that determine the shape of the head skeleton is lacking, recent studies strongly suggest that reciprocal interactions between NCCs and surrounding tissues are certainly important.

THE RIDDLE OF THE TYMPANIC MEMBRANE

For understanding the mechanism of ectomesenchymal specification, tissue interactions between the crest-derived ectomesenchyme and the ectodermal and endodermal epithelium should be taken into consideration. In this connection, it should be noted that comparative morphologists have also focused on a nonskeletal epithelial structure, the tympanic membrane, to resolve the puzzle.

The tympanic membrane separates the middle ear cavity (originally the position of the first pharyngeal pouch) from the external auditory meatus (originally the position of the first pharyngeal cleft; Fig. 1C–F). The nonmammalian tympanic membrane is attached to the quadrate, the upper jaw element (Fig. 1C, E). By contrast, in the mammalian middle ear, the tympanic membrane spans the ectotympanic, the angular homologue belonging to the lower jaw domain (Fig. 1D, F). In the old concept of middle ear evolution, "primitive" tetrapods, such as *Ichthyostega*, established the tympanic membrane at the quadrate position. This was carried over to extant reptiles and birds, and the mammalian middle ear evolved from the reptilian state (reviewed by Laurin, '98; Clack and Allin, 2004). The homology between the tympanic membranes of mammals and nonmammalian amniotes was based on this evolutionary concept for many years.

Goodrich ('14, '30) assumed that the mammalian tympanic membrane is homologous with that of reptiles, namely, that the reptilian tympanic membrane moved ventrally, resulting in its lower jaw position in mammals. By contrast, Gaupp ('11a,b, '12) doubted the homology because of the aforementioned topographical discrepancy in the attachment site of the tympanic membrane (Fig. 1C–F). His perspective seems to have been corroborated by detailed analyses of comparative embryology. Goodrich's theory is inconsistent with the topographical relationships between the tympanic membrane and muscles that arise in association with the malleus (m. tensor tympani and m. tensor veli palatine). These muscles develop as a pair of tandem anlagen that differentiate from a single primordium occupying a position equivalent to m. pterygoideus in nonmammalian amniotes (see also Barghusen, '86). Given that these muscles are homologous and that the connectivity of this muscle to the skeletal elements is conserved during the ventral translocation of the tympanic membrane as predicted by Goodrich, we would expect m. tensor tympani to be located ventral to the Eustachian tube (Fig. 6A).

However, this muscle is *dorsal* to the Eustachian tube of the mammalian middle ear (Fig. 6B). This morphological inconsistency apparently denies the homology of tympanic membranes between mammals and nonmammalian amniotes (see also Presley, '84).

Because of this topographical discrepancy, Westoll ('43, '44, '45) assumed the existence of a ventral diverticulum of the middle ear cavity called the recess mandibularis, which grows ventrally to form a mammal-specific tympanic membrane (Fig. 6C). The mammalian tympanic membrane is thus assumed to consist of a small dorsal part, corresponding to the reptilian tympanic membrane, pars flaccida, and a large ventral portion corresponding to a novel mammalian middle ear feature: pars tensa (Fig. 6C, D). Importantly, Goodrich ('14, '30) also pointed out that the mammalian middle ear cavity seems to protrude ventrally compared with nonmammalian amniotes with respect to the course of the chorda tympani, a branch of the facial nerve (Fig. 6E). In comparative anatomy, chorda tympani is regarded as a branch of the posttrematic ramus of the facial nerve (Fig. 6E). However, the mammalian chorda tympani looks deceptively like a branch of the pretrematic ramus of the facial nerve (Fig. 6E). Goodrich ('14, '30) assumed that the middle ear cavity swells ventrally only in mammalian embryos and that the chorda tympani is elevated from the ventral position by the swollen cavity (Fig. 6E). However, there is a competing theory on this issue. As the chicken chorda tympani is formed as a branch of the pretrematic ramus, the chorda tympani does not necessarily occupy the same position relative to the surrounding anatomical elements in all amniotes (Kuratani et al., '88). For this reason, it seems unreasonable that the course of the cranial nerves should be considered in comparative morphological analysis.

At any rate, because Westoll's theory ('43, '44, '45) persuasively explained the transitional state of the middle ear from reptilian to mammalian, several morphologists supported this theory for many decades (Gregory, '51; Watson, '53; Shute, '56; Parrington, '79). However, this concept of the origin of the middle ear requires radical revision in the light of recent paleontological evidence. One of the rationales for the old concept that the middle ear evolved in ancestral tetrapods was the presence of the "otic notch" in their skulls. The notch was once thought to be the site at which the tympanic membrane made contact with the stapes (Watson, '51; Romer, '66). However, many of the otic notches are now interpreted as spiracular notches rather than as hosts for the tympanic membrane, and the presence of the middle ear is no longer considered to be the ancestral condition of tetrapods (Clack, '89, '93; Brazeau and Ahlberg, 2006). Furthermore, the hyomandibular of "primitive" amniotes is massive and strut-like and there does not seem to be any room for the tympanic membrane in their skulls (Romer and Price, '40). Thus, the modern consensus is that the middle ears of mammals and nonmammalian amniotes developed independently of each other after their divergence from a
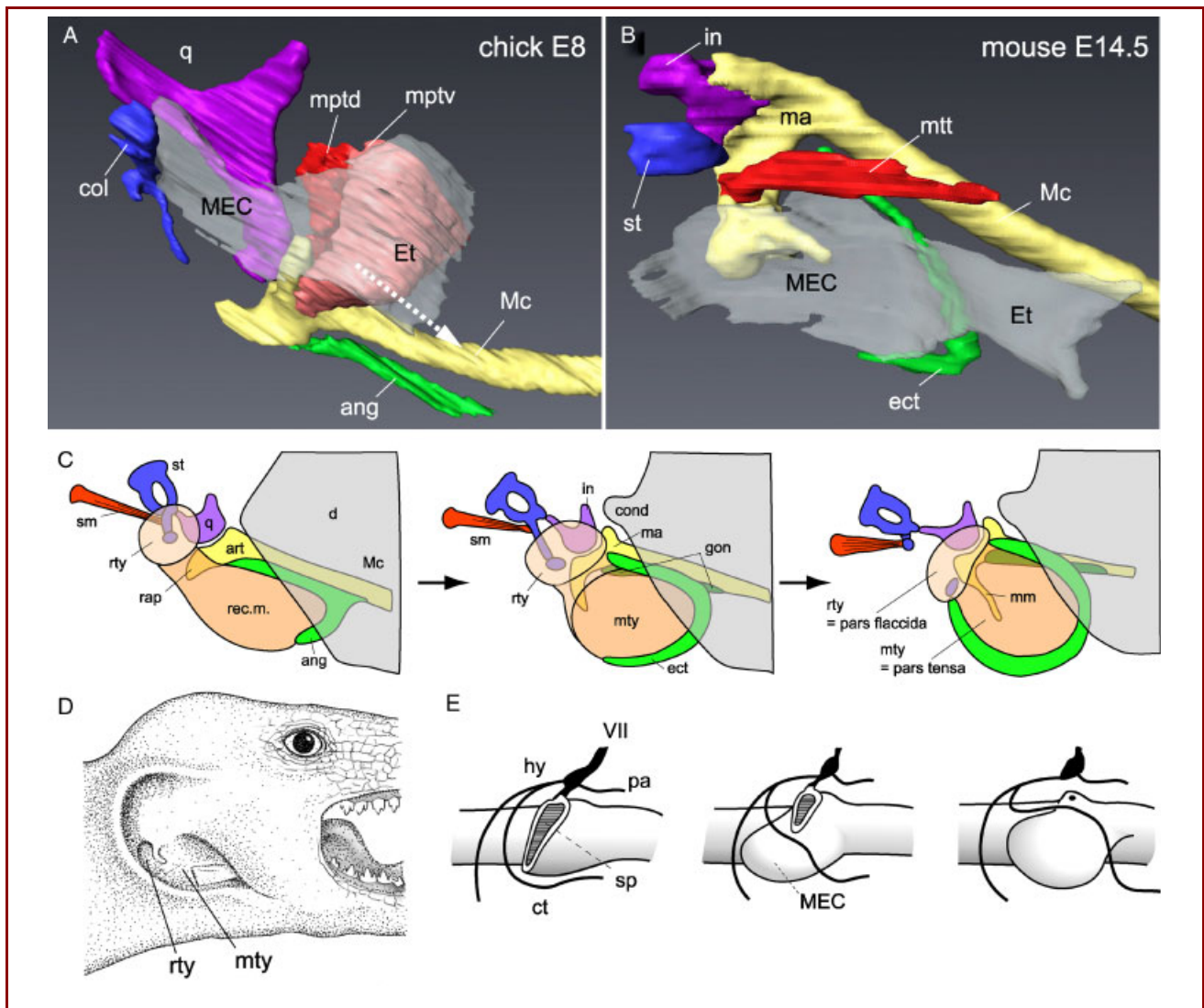
Figure 6. Interpretations of mammalian tympanic membrane evolution proposed by comparative morphologists. (A, B) Medial views of the primary jaw joint with middle ear cavities and some of the pterygoid muscle derivatives in chicken and mouse embryos. The chicken, m. pterygoideus (mptd and mptv), is located in the ventral region of the Eustachian tube (A). If the tympanic membrane is homologous between mammals and nonmammalian amniotes, m. tensor tympani, homologous with m. pterygoideus, should be located ventral to the Eustachian tube after the tube moved to the ventral position (predicted by Goodrich, '14, '30; arrow with a dotted line). However, in the mouse embryo, m. tensor tympani is located dorsal to the Eustachian tube (B), suggesting that the tympanic membrane is not homologous between mammals and nonmammalian amniotes. (C) Diagrams showing the "recess mandibularis theory" put forward by Westoll ('43, '44, '45). Three suggested stages of mammalian middle ear evolution from an advanced theriodont (a group of therapsids) viewed from the lateral side. The functional part of the mammalian tympanic membrane, pars tensa, is considered to be a novel structure that arose by contact between a novel diverticulum (the recess mandibularis; rec. m.) formed in the ventral position of the original middle ear cavity and the outer skin. Pars flaccida is believed to be a vestigial reptilian tympanic membrane. (D) Lateral view of the head of the reconstructed *Thrinaxodon* based on Westoll's theory (Allin and Hopson,'92). (E) The evolutionary transitions from fish to mammals (left to right) in the course of chorda tympani according to Goodrich's theory ('14, '30). The anterior is to the right. Although chorda tympani is generally thought to be a branch of the posttrematic ramus of the facial nerve (the hyoid ramus; hy), it looks deceptively like a branch of the pretrematic ramus of the facial nerve (the palatine ramus; pa) in mammals. Goodrich assumed that the mammalian middle ear cavity does not swell laterally but ventrally, and that it pushes chorda tympani to the anterior, which is why the mammalian chorda tympani looks deceptively like a branch of the pretrematic ramus.

common ancestor (Lombard and Bolt, '79; Laurin, '98; Clack, 2002a,b; Müller and Tsuji, 2007). Theories in which it was assumed that the mammalian middle ear evolved from the reptilian state of the middle ear, as typified by Westoll, do not seem to be borne out by the paleontological evidence.

PERSPECTIVES—THE TYMPANIC MEMBRANE AND SECONDARY JAW JOINT

Although Westoll's theory ('43, '44, '45) has been superseded, the origin of the mammalian tympanic membrane remains to be explained and seems to be the most important remaining issue in mammalian middle ear evolution. In this context, elucidation of the connections and release of connections among the first and second arch elements is important. The incus is in contact with the stapes in mammals, which represents hyostylic connectivity (Fig. 1D, F). By contrast, the quadrate and columella auris are separate from each other in nonmammalian amniotes, which illustrates the release of the ancestral hyostylic connectivity (Fig. 1C, E). In mammals, the middle ear has taken over the primitive hyostylic connectivity that can be observed in sharks and its skeletal complex has been released from the original task of the jaw, which was probably facilitated by the establishment of the secondary jaw joint between the squamosal and dentary (Fig. 2). Thus, it is very likely that mammalian ancestors experienced changes in the shark-like developmental program that resulted in a shift of the skeletal elements and the development of a sound-transmitting apparatus from the first and second pharyngeal arches. Nonmammalian amniotes evolved the middle ear as a result of a different set of changes in the shark-like developmental program that resulted in a shift of a different set of skeletal elements.

Analyses of paleontological data suggest that these morphological changes are closely related to the anatomical position at which the tympanic membrane arose. In nonmammalian amniotes, the hyomandibular was released from the quadrate and the tympanic membrane evolved at a region intermediate between the two elements. Changes in mammalian ancestors seem to have been more complicated. The reflected lamina of the angular (Fig. 2A) was once thought to be the attachment for the pterygoid muscles (e.g., Romer and Price, '40; Barghusen, '68). However, Allin ('75) pointed out that the skeletal configuration of many therapsids does not favor this concept and claimed that the lamina served as an attachment site for the tympanic membrane (see also Sushkin, '27; Allin, '86; Allin and Hopson, '92; Clack and Allin, 2004), consistent with comprehensive analyses of the tetrapod ear (Lombard and Bolt, '79). Based on these assumptions, it can be hypothesized that airborne sound was transmitted into the inner ear through the angular–articular–quadrate–hyomandibular route in the early stage of mammalian evolution (possibly in the early therapsids, such as *Biarmosuchia*; Allin and Hopson, '92) and that selective forces directed toward more efficient auditory function decreased the size of these elements.

For this reason, the establishment of the tympanic membrane in the angular position would have been a prerequisite for morphological changes in the skeletal elements of the mammalian middle ear (Clack and Allin, 2004).

From the above discussion, the most important question to be solved seems to be how the tympanic membrane formed in the lower jaw domain in the mammalian ancestor. To address this issue, we have to focus on evolutionary changes in pharyngeal arch developmental programs by comparative studies at a molecular genetic level. To this end, it is first necessary to identify comparable developmental stages in mammals and nonmammalians. Our preliminary observations suggest that the skeletal elements of the primary jaw joint start to form at different positions of first arches in the mouse and chick embryos, indicating that similar stages might be identified in embryos in which prechondrogenic condensations in pharyngeal arches have not been observed (Takechi and Kuratani, unpublished data). Based on the identification of these stages, it should be possible to describe in developmental and molecular terms the critical developmental events that result in the mammalian tympanic membrane in the lower jaw. Given that the ventral swelling of the middle ear cavity, predicted by Westoll and Goodrich, has not been verified in mammalian development, we should determine which developing tissues (e.g., the prechondrogenic condensations, middle ear cavity, and external auditory meatus) shift along the anteroposterior, dorsoventral, or mediolateral axes in mammals and nonmammalians. In this regard, developmental signals involved in the "shape" of the skeleton, namely, ectomesenchymal specification, endodermal instruction, and autonomous roles for skeletal shaping in NCCs, should be considered to elucidate the shift that took place in mammalian evolution. We also note that recent genetic analyses in the mouse have provided information about genes central to middle ear formation (reviewed by Mallo, '98, 2001, 2003; Fekete, '99). Of these, *Goosecoid* mutant mice showed deficiencies of the malleus manubrium, processus brevis and the ectotympanic and tympanic membrane in the middle ear region (Rivera-Perez et al., '95; Yamada et al., '95; Kuratani et al., '99). The double knockout of *Msx1* and *Msx2* resulted in the absence of the malleus manubrium and the processus brevis, and incomplete tympanic membrane development (Zhang et al., 2003). These results strongly suggest that some of the NCC-derived lower jaw elements and the tympanic membrane exhibit an interdependent relationship in the mammalian developmental program. It is important to understand how the ectomesenchyme interacts with the epithelial structure in nonmammalian middle ear development.

The establishment of the secondary jaw joint is also an important issue in mammalian middle ear evolution and its evolution seems to be related to the *Otx2* expression pattern (reviewed by Kuratani et al., '97). *Otx2* is expressed in NCCs derived rostral to the boundary between the midbrain and the

hindbrain, and some of the *Otr2*-positive NCCs flow into the first arch (Fig. 5A). In the *Otr2* heterozygous knockout mouse, only the dentary exhibited a graded series of deficiency (from almost normal-to-absent), whereas the postdentary elements were normal (Matsuo et al., '95). This phenotype seems to be complementary to that of the *Hoxa-2* mutant mouse in respect of the first arch skeleton. In the *Hoxa-2* mutant, only the postdentary component of the first arch skeleton is duplicated (Fig. 5B), whereas in the *Otr2* heterozygous mutant, only the dentary is lost. It has been reported that NCCs in the first arch originating from the rhombencephalic and mesencephalic regions do not seem to intermingle but are spatially dissociated from each other within the first arch (Le Lièvre, '74; Osumi-Yamashita et al., '94, '96; Imai et al., '96; Köntges and Lumsden, '96). Thus, the first arch ectomesenchyme seems to be a composite structure, consisting at least of the Hox-default proximal and the distal *Otr2*-dependent part. Separation of the compartments seems to have facilitated independent evolution in the mammalian lineage, as the size of the postdentary elements was reduced and that of the dentary was increased. Detailed comparisons of developmental changes in *Otr2*-positive NCCs between mammals and nonmammalian amniotes should resolve the issue of the establishment of the secondary jaw joint.

Mammalian middle ear evolution has attracted the attention of morphologists and anatomists for many years. This issue is a good example of the rigidity and flexibility of the vertebrate pharyngeal developmental system. We believe that a comprehensive description of mammalian middle ear evolution can be developed using extant animals and the perspectives outlined in this review.

ABBREVIATIONS

ang	angular bone
art	articular bone
ch	ceratohyal
col	columella auris
cond	condyle process
ct	chorda tympani
d	dentary bone
EAM	external auditory meatus
eh	epihyal
ecol	extracolumella
ect	ectotympanic bone
Et	Eustachian tube
gon	goniale
hm	hyomandibular
hy	hyoid ramus of the facial nerve
in	incus
inc	intercalary
iph	infrapharyngohyal
lh	laterohyal
ma	malleus
Mc	Meckel's cartilage
MEC	middle ear cavity
mm	malleus manubrium
mptd	m.pterygoideus dorsalis
mptv	m. pterygoideus ventralis
mtt	m. tensor tympani
mty	mammalian tympanic membrane
n	notchode
oc	otic capsule
PA	pharyngeal arch
pa	palatine ramus of the facial nerve
pb	processus brevis
pq	palatoquadrate bone
pt	pterygoid
q	quadrate bone
qj	quadratojugal bone
rap	retroarticular process
Rc	Reichert's cartilage
rec.m.	recess mandibularis
rl	reflected lamina
rty	reptilian tympanic membrane
sa	surangular bone
sh	stylohyal
sm	stapedial muscle
sp	spiracle
sph	suprapharyngohyal
sq	squamosal bone
st	stapes
sty	styloid process
ty	tympanic membrane
VII	facial nerve

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