Evolutionary Development and Homoplasy of the Middle Ear in Early Mammal Evolution

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About the Author

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Representative Articles

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Abstract

To understand the origins of complex structures and evolutionary novelties is a central quest in evolutionary studies. A key innovation in the early evolution of mammals is in the middle ear, a significant adaptation to sensitive hearing. The Definitive Mammalian Middle Ear (DMME), a major apomorphy of modern mammals, is defined by the loss of the embryonic Meckel's cartilage that connects the middle ear to the mandible in early ontogeny. The resorption of this cartilage in the adult separates the middle ear from the mandible, and this separation enhances the auditory function of the ear and the feeding

function of the jaws. On the broadest scale of 310 million years of evolution of mammals from pre-mammalian synapsid ancestors, transformation of the middle ear and its precursory structures appears to be incremental and orderly. However, recent discoveries of new fossils have demonstrated major evolutionary convergences and reversals, also known as homoplasy, in the middle ear structure among Mesozoic mammal clades. Some homoplasies of the middle ear structures in the Mesozoic mammals can now be correlated with heterochrony, or timing changes in ontogeny. Other homoplasies show the same phenotypic characteristics of gene patterning in the embryogenesis of extant mammals. Recent discoveries in new fossils and in developmental genetics have added to a growing body of evidence that the seemingly labile evolution of the middle ears in Mesozoic mammals was influenced, in a major way, by developmental heterochrony, and gene patterning. Changes in developmental genes and ontogenetic timing are a driving mechanism for the evolution of complex structures with major biological functions, as seen in the early evolution of mammalian ears.

Key Words

Evolution; development; gene patterning; mammal middle ear

Introduction

Mammals are distinctive from non-mammalian vertebrates by many evolutionary innovations that have important biological adaptations. Transformation of these derived features occurred in the 310 million years of evolution of mammals from the premammalian synapsid ancestry, by the diversification of the basal lineages of mammals during the Mesozoic (Kielan-Jaworowska *et al*. 2004; Kemp 2005; Luo 2007). One derived feature of mammals is their sophisticated middle ear with greatly enhanced hearing function. The sophisticated ears may have facilitated the diversification of mammals in the dinosaur-dominated Mesozoic terrestrial ecosystem, presumably in the nocturnal niches, by the enhanced hearing capability in the darkness of the night (Allin and Hopson 1992; Manley 2000; Kielan-Jaworowska *et al*. 2004; Luo 2007). The evolutionary novelties in the ear of Mesozoic mammals led to spectacular functional adaptations in hearing in Cenozoic mammals, from echolocation to infrasonic long-distance hearing, to communication in many species, such as languages of humans (Fleischer 1973; Durrant and Lovrinic 1984; Simmons *et al*. 2008; Veselka *et al*. 2010).

The mammalian middle ear consists of the ecto-tympanic ring for the tympanic membrane (the ear drum), the malleus (the hammer bone), the incus (the anvil bone), and the stapes (the stirrup bone). In the extant mammals, the anterior part of the malleus, embryonically developed from a separate ossification, known as the goniale, is connected to the ectotympanic both in embryo and in adult, and to the Meckel's cartilage in embryo. The ectotympanic, the malleus, the anterior (goniale) element of the malleus, and the incus of mammals are homologues to the angular bone, the articular bone, the prearticular bone, and the quadrate of non-mammalian vertebrates including premammalian synapsid relatives to mammals, such as the Triassic cynodont *Thrinaxodon* (Fig. 1A-C).

Vertebrate morphologists and embryologists of the 19th century first recognized that the embryonic Meckel's cartilage of the first gill arch of gnathostome vertebrates is the embryonic precursor both to the malleus and the incus of the middle ear in mammals, and to the quadrate and the articular of other vertebrates (Reichert 1837; Gaupp 1913; see a recent review by Takechi and Kuratani 2010). The quadrate and the articular form the primary jaw joint in all non-mammalian gnathostome vertebrates; in premammalian cynodonts, the articular-quadrate joint (Fig. 1A: "primary" A-Q joint) serves as the jaw joint for feeding, in addition to receiving sound for hearing.
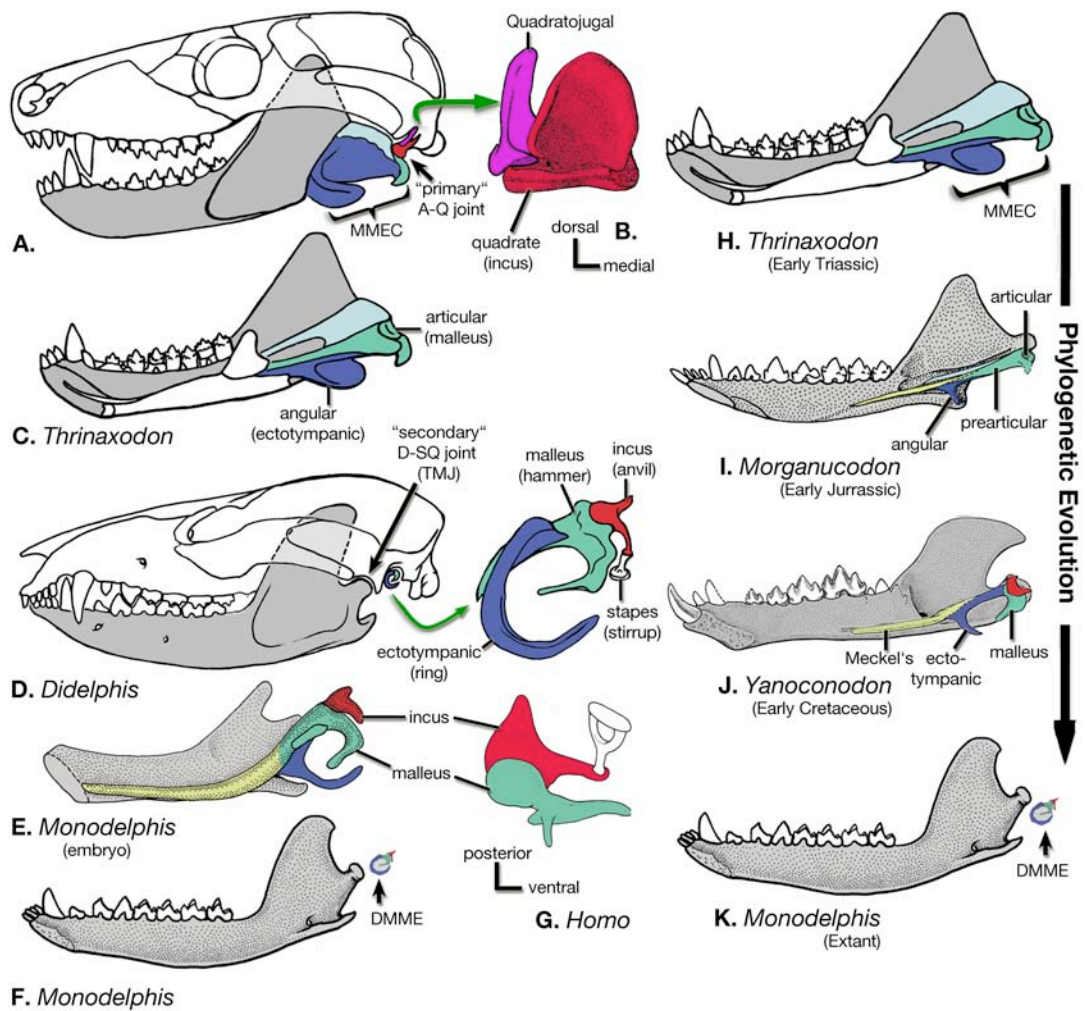
▲ Fig. 1

Homology and evolution of the middle ears from the premammalian cynodonts to extant mammals. A-C, Cynodont *Thrinaxodon*: skull (A – lateral view), the quadrate (incus) and quadratojugal bones (B – posterior view), the mandible and the Mandibular Middle Ear of Cynodonts (MMEC) (C – medial view). D-F, Extant marsupial mammals: adult skull (D – lateral view), embryonic mandible and middle ear connected by Meckel's cartilage (E – medial view); adult Definitive Mammalian Middle Ear (DMME) separated from the mandible (F – medial view). G, *Homo*, the middle ear bones of malleus (hammer), incus (anvil) and stapes (stirrup) (internal view, rotated 90° counter-clockwise for the incus to be in the same orientation as it homologous quadrate of cynodonts in Fig. 1B). Phylogenetic transformation series of mandibles and middle ears (medial view): H. *Thrinaxodon* (Early Triassic, about 250 million years), I. *Morganucodon* (Earliest Jurassic, 200 million years), J. *Yanoconodon* (Early Cretaceous, 125 million years), and K. *Monodelphis* (extant). Figures not on same scale. A and F original; others adopted from Luo (2007), Luo *et al*. (2007a), and Ji *et al*. (2009).

The stapes of mammals is homologous to the columella of all tetrapod vertebrates (Romer and Parson 1986). This element of the middle ear did not show much change in the early evolution of mammals from cynodonts (Allin and Hopson 1992: Kielan-Jaworwoska *et al*. 2004). However, other elements of the middle ear have undergone a fundamental transformation.

In all pre-mammalian cynodonts and most mammaliaforms, the middle ear bones are fully attached to the mandible. The angular bone is plate-like and has a large surface area for receiving sound (Allin 1975; Allin and Hopson 1992); together with the articular (malleus), it forms the Mandibular Middle Ear of Cynodonts (Fig. 1C: MMEC). By contrast, the mammalian malleus and incus, the homologues of the quadrate and articular in cynodonts, are connected to the mandible by the Meckel's cartilage in embryonic and fetal stages, but separated from the mandible later in the adult (Fig. 1E-F). The size of middle ear bones is very small and well adapted for hearing, but they are detached from the jaw joint in the adult and have no function for feeding. The separation of the middle ear bones from the mandible enhances the auditory function of the ear and the feeding function of the jaws (e.g., Crompton and Parker 1978).

The mammalian jaw joint, also known as the temporomandibular joint (Fig. 1D: TMJ), is formed by the dentary and the squamosal. This "secondary" jaw hinge replaces the "primary" jaw joint between the quadrate (incus) and the articular (malleus) as the functioning hinge for the jaws (A-Q joint). In several advanced pre-mammalian cynodonts or mammaliaforms of the Triassic and Early Jurassic, the primary "reptilian" jaw joint of the articular and the quadrate coexists, side-by-side, with the secondary and evolutionarily derived dentary-squamosal joint (D-SQ joint). In extant mammals, the articulation between the dentary and the squamosal becomes the only jaw joint after the middle ear is disconnected from the lower jaw by the resorption of the embryonic Meckel's cartilage. Reduction in the size of middle ear bones, and loss of the embryonic Meckel's cartilage through ontogeny of extant mammals are parallel to the pattern of cynodont-mammal phylogenetic evolution.

In phylogenetic evolution (Fig. 1H-K), the mandibular middle ear of cynodonts (MMEC) gradually decreases in size, and becomes increasingly more gracile for a more sensitive detection of sound, in the successively more derived groups that are closer to modern mammals (Allin 1975; Allin and Hopson, 1992; Luo and Crompton 1994; Rowe 1996; Sidor 2001). This eventually culminated in the definitive mammalian middle ear (DMME), which is exclusively suspended in the base of the cranium, therefore also known as the "cranial middle ear" (Rowe 1996; Luo *et al.* 2007a).

On the broadest phylogenetic scope from Paleozoic synapsids to modern mammals, the evolution of the mandibular and middle ear structures appears to be incremental, and suggests a stepwise assembly of an integrated character complex (Crompton 1972; Allin 1975; Luo 1994; Sidor 2001; Kemp 2005). As can be mapped on the cynodont-mammal phylogeny (e.g., Kemp 2005; Luo 2007), the evolution of the mammalian mandible and the ear is incremental, and can be best seen in the enlargement of the dentary bone, at the expense of other bony elements in the mandible in advanced cynodonts (Crompton 1972), resulting in the establishment of the dentary/squamosal jaw joint for more forceful mastication (Crompton and Hylander 1986), the miniaturization of the middle ear (Allin 1975), and the stepwise transformation of the quadrate (incus) toward a more mobile suspension from the cranium (Luo and Crompton 1994). The incremental evolution in the entire mandible and middle ear has been also demonstrated by morphometric analyses of their characteristics in the time-calibrated phylogeny (Sidor and Hopson 1998; Sidor 2001).

The extant monotreme, marsupial and placental mammals have all lost the embryonic Meckel's cartilage in the adult, and have all achieved the definitive mammalian middle ear (DMME). If these living lineages were the only mammals to be sampled, without reference to the fossil record of Mesozoic mammals, then the separation of

the middle ear from the mandible would appear to have evolved only once, in a singular evolutionary origin for extant mammals (Kemp 1983; Rowe 1988). However, others have suggested that the middle ears have evolved convergently in mammals (Hopson and Crompton 1969; Hopson 1991; Rich *et al*. 2005).

The definitive mammalian middle ear (DMME) is a complex structure. Whether this key feature had a singular origin, or evolved multiple times, has a broader bearing on how complex structures evolve in general. Besides its direct bearing on the mammalian origins, it is relevant to two perspectives on evolution. First, would a more complex structure be less labile in evolution on parsimony principle and therefore less susceptible to homoplasy (Martin and Luo 2005)? Because the mammalian middle ear is an intricate structure, is it less labile in the early evolutionary history of mammals, therefore a more reliable character for morphological systematic studies?

Secondly, the morphological disparity and lineage diversity are far greater in Mesozoic mammals, than could be inferred from living mammals alone. Extant monotremes, marsupials and placentals all have their separate evolutionary lineages extending into the Mesozoic. Although marsupials and placentals later diversified in the Cenozoic after the extinction of dinosaurs and surpassed all of their distant mammalian relatives in the Mesozoic (Rose 2006), these extant mammals are descendants from only three Mesozoic lineages, out of more than 20 other mammal lineages that became extinct in the Mesozoic (Kielan-Jaworowska *et al*. 2004; Benton 2005). The extinct Mesozoic mammalian clades have showed many (although not all) modern mammalian ecological specializations (Luo *et al*. 2003; Luo and Wible 2005; Ji *et al*. 2006; Meng *et al*. 2006; Martin 2006). Moreover, some Mesozoic mammals exhibit a wider range of morphological disparity in dental morphology (Luo *et al*. 2001a; 2007b), in limb structure (Luo and Wible 2005; Meng *et al*. 2006), in paedomorphic retention in the adult of the embryonic condition in extant mammals (Wang *et al*. 2001; Li *et al*. 2001; Meng *et al*. 2003; Li *et al*. 2003; Ji *et al*. 2009). How can evolutionary morpholo-

gists use the greater structural complexity of fossils from deep geological times, to better inform the overall evolutionary history of both modern and fossil mammal groups?

1. Patterns in Phylogeny

1.1 Primitive condition in cynodonts and mammaliaforms

In several advanced cynodonts and primitive mammaliaforms of the Triassic and the Early Jurassic, the primary "reptilian" jaw joint of the articular (malleus) and the quadrate (incus) co-exist, side-by-side, with the secondary and evolutionarily derived jaw joint of the dentary and the squamosal. The precursory condition to the fully functional dentary-squamosal jaw hinge occurred in premammalian cynodonts *Pachygenelus* (Crompton 1972; Allin and Hopson 1992) and *Brasilodon* (Bonaparte *et al*. 2005; Luo 2007). These groups are more derived than other premammalian cynodonts in having the dentary-squamosal contact.

The fully formed jaw joint of the dentary condyle and the squamosal glenoid with load-bearing function is present in *Sinoconodon*, *Morganucodon* and docodonts. In these "transitional" groups, the joint between the quadrate (incus) and the articular (malleus) serves the dual function for jaw hinge and for transmitting sound received by the malleus (articular) and the ectotympanic (angular), which are solidly attached to the mandible (Kermack *et al*. 1981; Lillegraven and Krusat 1991; Crompton and Luo 1993; Ji *et al*. 2006) (Fig. 2H-I).

The mandibular middle ear of cynodonts (MMEC) is held in the postdentary trough and to the Meckel's groove on the medial side of the mandible, in advanced cynodonts and majority of mammaliaforms (Fig. 1H-I). Because the postdentary trough and the Meckel's groove are preserved in many more fossils than the more delicate ear bones themselves, the presence of the postdentary trough provides a reliable inference that the middle ear must have been attached to the mandible, even if the ear bones themselves are not preserved in incomplete fossils. The

Meckel's sulcus on the mandible accommodates the Meckel's element, which is continuous with the prearticular bone in mammaliaforms (Fig. 1I). The prearticular is the homologue to the goniale element of the malleus in the extant mammals (Zeller 1989). In some Mesozoic mammals, the Meckel's groove on the mandible still holds the ossified Meckel's cartilage that is connected to the middle ear, even without the postdentary trough (more information below).

Hadrocodium from the Lower Jurassic of Yunnan, China, is the earliest-known mammaliaform that has lost both the postdentary trough and the Meckel's groove (Fig. 3B). Because the mandibular structures holding the middle ear are completely lost, it is hypothesized that in *Hadrocodium*, the middle ear bones were already fully separated from the mandible (Luo *et al*. 2001b; Kielan-Jaworowska *et al*. 2004).

1.2 Middle ears of major Mesozoic mammal groups

The Mammalia, or the crown mammals, is a monophyletic group defined by the last common ancestor of monotremes, marsupials and placentals (Rowe 1988). This common ancestor is at least 170 million years old and no younger than the Middle Jurassic. Many Mesozoic groups of mammalia are extinct orders or families that did not give rise to any Cenozoic mammals (Kielan-Jaworowska *et al*. 2004; Luo 2007). Nonetheless they are phylogenetically nested in the mammalia, by parsimony of osteological and dental evidence. Three extinct mammalian orders are very abundant in the Jurassic and Cretaceous: eutriconodonts, multituberculates, and spalacotheroids, which are successively closer to the living therians, than to the lineage of monotremes (Kielan-Jaworowska *et al*. 2004).

1.2.1 Monotremes and their kin

The middle ear is fully separated from the mandible in the adults of modern egg-laying monotremes (Kuhn 1971; Zeller 1989). In embryos, the Meckel's cartilage is present, and connects the precursors of the malleus and the ectotympanic to the mandible. But in neonates, after the Meckel's cartilage is reabsorbed, the middle ear becomes separated from the mandible in the adult (Fig. 2F-H). One interesting characteristic of monotremes is that their Meckel's cartilage and middle ear bone precursors are displaced medially from the mandible in embryos; this medially displaced position of the ear bones have remained the same in the adult, after the resorption of the Meckel's cartilage (Fig. 2F-G).

The monotreme lineage first appeared in the Early Cretaceous. The Cretaceous monotremes *Steropodon* and *Teinolophos* have preserved the Meckel's sulcus. This indicates, unambiguously, that their middle ear was still connected to the mandible in some ways. While the posterior part of the mandible is not preserved in the only fossil specimen of *Steropodon*, it is interpreted that *Teinolophos* has the postdentary trough, which would accommodate the mandibular middle ear, as seen in mammaliaforms. Therefore it is inferred that the middle ear bones would be solidly lodged in the mandible (Rich *et al*. 2005; Martin and Luo 2005). However, the presence of the postdentary trough in *Teinolophos* has been challenged (Bever *et al*. 2005; Rougier *et al*. 2005; Rowe *et al*. 2008). In despite of the controversy (Rowe *et al*. 2008; Philips *et al*. 2009), in my view, the current evidence favors that *Teinolophos* retained the mandibular attachment of the middle ear (see also Hopson *et al*. 2009).

The crown group of monotremes is nested in the larger australosphenidan group (Luo *et al*. 2001a; Martin and Rauhut 2005; Rougier *et al*. 2007; Phillips *et al*. 2009). The several australosphenidan mammals preserved with mandibles show both the Meckel's groove, and the postdentary trough (Rich *et al*. 1997; Martin and Rauhut 2005; Rougier *et al*. 2007). It is clear that ancestrally the middle ear must have attached to the mandible in the australosphenidan-monotreme clade. The separation of the middle ear from the mandible must have evolved in extant monotremes after the australosphenidan-monotreme clade was split from other mammals. The definitive mammalian middle ear (DMME) would be independently acquired after the monotreme lineage had diverged from other major mammalian lineages (Rich *et al*. 2005) (Fig. 3: Hypothesis A).
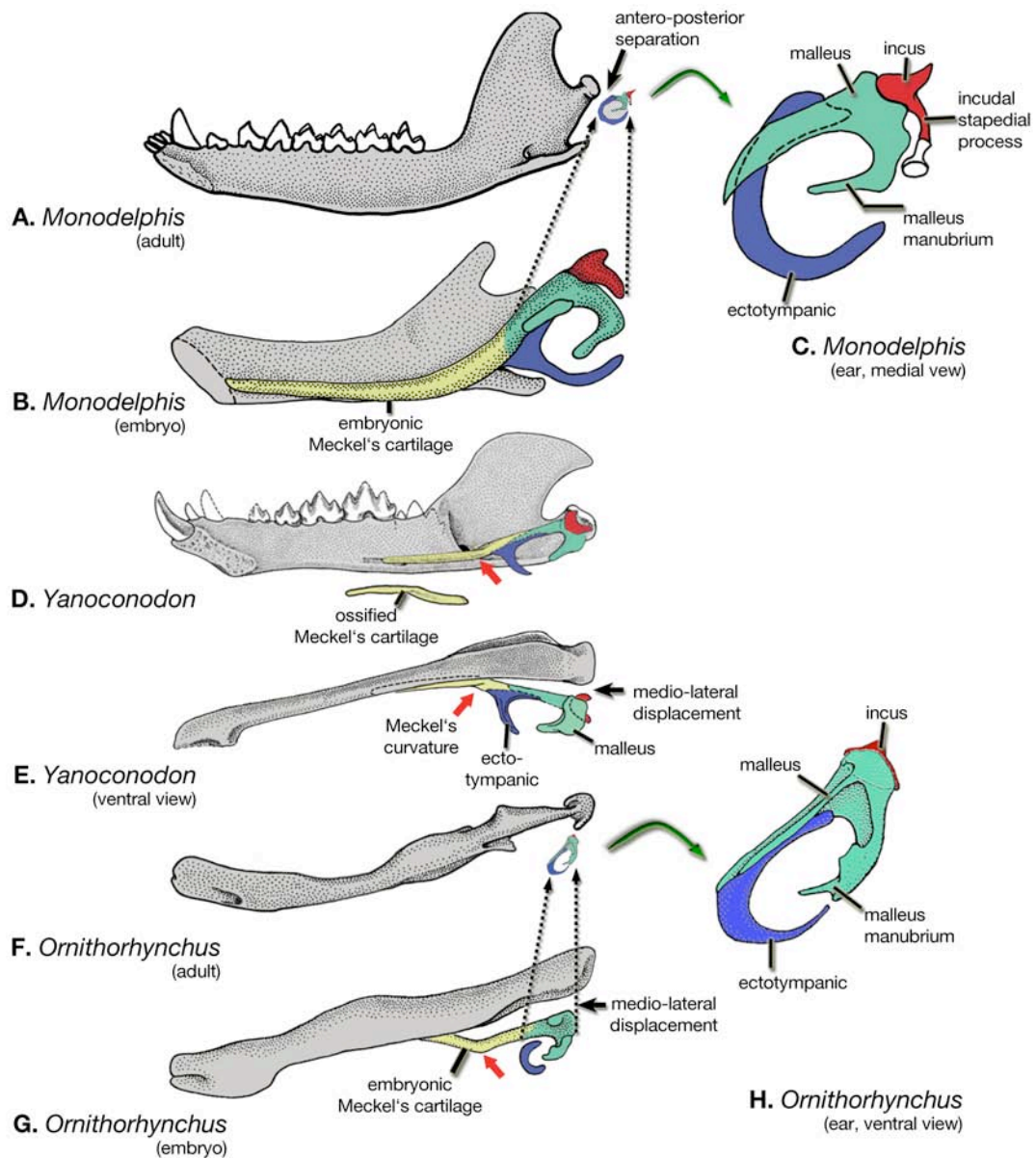
▲ Fig. 2

Allometry and development of the middle ears in extant mammals and in the Mesozoic mammal *Yanoconodon*. A-D, mandibles and middle ear (medial view); E-H, mandible and middle ear (ventral view). Dash lines: negative ontogenetic allometry of middle ear elements relative to the skull and mandible in extant marsupials and monotremes. The large and fully ossified middle ear in *Yanoconodon* suggests that the typical negative allometry, as seen in normal development of extant mammals, did not occur in *Yanoconodon*; or the middle ear bones in *Yanoconodon* must have sustained growth in late ontogenetic stage to a large size in adult, relative to the mandible. The ossified Meckel's cartilage of *Yanoconodon* is similar, in morphology, to the embryonic Meckel's cartilage of extant mammals; this Mesozoic mammal shows a paedomorphic retention of the embryonic condition of extant mammals. Figures not on the same scale, adopted from Luo *et al.* (2007a) and Luo (2007).

1.2.2 Eutriconodonts

Eutriconodonts are insectivorous to carnivorous mammals, characterized by molar teeth with three cusps in a straight line. This group is entirely extinct; it ranges from the Middle Jurassic to the Late Cretaceous and has a distribution in both the Laurasian and Gondwanan landmasses (Kielan-Jaworowska *et al.* 2004). On the evolutionary tree of all Mesozoic and extant mammals, eutriconodonts are placed closer to therians than monotremes, inside the crown group of Mammalia (e.g., Luo *et al.* 2007a; Gao *et al.* 2009). Gobiconodontids and jeholodontids of the Early Cretaceous of China are two eutriconodont families preserved with the ossified Meckel's cartilage (Fig. 3D). The discoveries of Meckel's cartilage in the gobiconodontids *Repenomamus* and *Gobiconodon* were an important breakthrough and showed that the middle ear are still connected to the mandible (Li *et al.* 2000; Wang *et al.* 2001; Meng *et al.* 2003; Li *et al.* 2003). It suggests that, although the embryonic Meckel's cartilage is reabsorbed in adult in all living mammals, this cartilage was ossified and permanently retained in adults of some Mesozoic mammals.

The jeholodontid *Yanoconodon* adds further evidence on the ectotympanic and the malleus that are connected to the ossified Meckel's cartilage. Its ectotympanic and malleus are large relative to the size of the cranium and the mandible (Fig. 2D-E). Given the larger size and the orientation of the middle ear bones, the articulation of the middle ear by the incus to the cranium is at the same level (in a "co-axial" alignment) as the temporomandibular joint of the dentary and the squamosal. This alignment made it feasible that movement at the dentary-squamosal jaw joint would not disrupt the middle ear for sound transmission. *Yanoconodon* has a Meckel's sulcus but no postdentary trough, but nonetheless its middle ear is still connected to the mandible through the ossified Meckel's cartilage. *Jeholodens* (Ji *et al.* 1999)*,* which is closely related to *Yanoconodon,* also has an ossified Meckel's cartilage, although loose from the mandible due to *post-mortem* dissociation (Luo *et al.* 2007a). While the presence of the postdentary trough would be sufficient for inferring the presence of the middle ear attach-

ment to the mandible, these eutriconodonts show that the absence of the postdentary trough could not exclude the possibility of the middle ear attachment to the mandible.

1.2.3 Multituberculates

Multituberculates are omnivorous to herbivorous mammals, characterized by several tooth cusps arranged in multiple rows on molars. The group has a long geological history from the Middle Jurassic to the Eocene, and are very abundant in some vertebrate fossil assemblages of the Late Jurassic and the Late Cretaceous. The middle ear bones were first discovered in the Late Paleocene multituberculate mammal *Lambdopsalis* in the 1980s, and corroborated by subsequent studies (Miao and Lillegraven 1987; Meng and Wyss 1995) (Fig. 3E). This was the first case for a Mesozoic mammal lineage to be found with the definitive mammalian middle ear, followed by further discoveries in the Cretaceous multituberculates *Chulsanbaatar* and *Nemegtbaatar* (Hurum *et al.* 1996; Rougier *et al.* 1996). In multituberculates, the middle ear bones are completely separated from the mandible, and are only attached to the cranium. The characteristics of the middle ear articulation with the cranium are very similar to the condition of extant monotremes (Meng and Wyss 1995; Hurum *et al.* 1996).

While the presence of the definitive mammalian middle ear in multituberculates is well-documented by fossils, its broader implications for mammalian evolution would depend on phylogenetic context. As initially proposed by Rowe (1988), multituberculates are more closely related to extant marsupials and placentals, than eutriconodonts; and multituberculates and derived therians are united in the theriiform clade. This phylogenetic position of multituberculates has been supported by the majority of later studies (Fig. 3E: node 4). Based on this phylogeny, the definitive mammalian middle ear would be a shared derived character of the theriiform clade and it is homologous in multituberculates and extant therians. However, some paleontologists have long contended that multituberculates are close relatives to the Late Triassic haramiyidans (e.g., Butler 2000). The Late Triassic haramiyidans
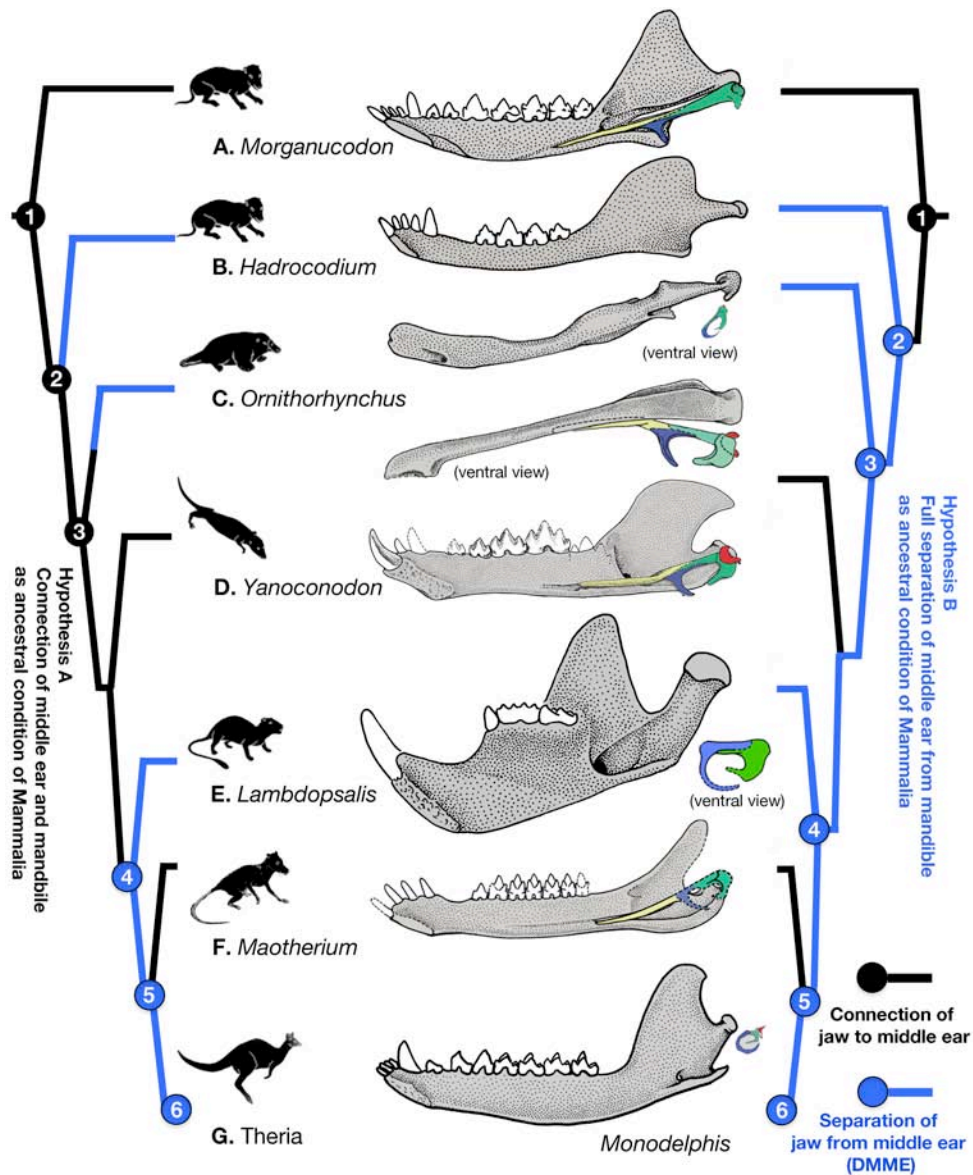
▲ Fig. 3

Homoplastic evolution of the Definitive Mammalian Middle Ear (DMME) in Mesozoic mammals by developmental heterochrony and gene patterning. A, mammaliaform *Morganucodon*; B, mammaliaform *Hadrocodium*; C, extant monotreme *Ornithorhynchus*; D, euticonodont *Yanoconodon*; E, multituberculate *Lambdopsalis*; F, spalacotheroid *Maotherium*; F, extant therians (such as kangaroo and *Monodelphis*). Node 1: Mammaliaforms; Node 2: Clade of *Hadrocodium* through extant therians; Node 3: Crown Mammalia; Node 4: Theriiform Clade (of multituberculates and extant therians); Node 5: Trechnotherian Clade (of *Maotherium* and other spalacotheroids through extant therians). Alternative hypothesis A - On the assumption that the connection of the middle ear to the mandible represents an ancestral condition of some mammaliaforms and crown Mammalia (Nodes 2, 3), the acquisition of the definitive mammalian middle ear must have evolved three times: in *Hadrocodium* (Luo *et al*. 2001b), in extant monotremes (Rich *et al*. 2005), and lastly in theriiforms (Meng and Wyss 1995; Hurum *et al*. 1996; Rougier *et al*. 2006); but this is reversed in spalacotheroid *Maotherium* within the theriiform clade (Ji *et al*. 2009). Alternative hypothesis B - On the assumption that a full separation of the middle ear from the mandible is the ancestral condition of the clade of *Hadrocodium* (node 2) through extant Theria Clade (node 6), the "re-connection" of the mandible to the middle ear must have evolved twice by reversal: in the euticonodonts *Gobiconodon*, *Repenomamus* and *Yanoconodon* by direct evidence, and in *Jeholodens* by inference, and in the spalacotheroid clade including *Maotherium* by direct evidence (Ji *et al*. 2009), and in *Zhangheotherium* by inference (Meng *et al*. 2003). Blue lines and nodes represent the derived evolutionary condition; whereas the black nodes and lines represent the primitive condition of the middle ear.

have the full complement of mandibular structures for housing the middle ear in the mandible (Jenkins *et al.* 1997). On this phylogenetic assumption (reviewed by Kielan-Jaworowska *et al.* 2004), the definitive mammalian middle ear would have evolved in the haramiyidan-multituberculate clade, after the clade had split from extant therians. The theriiform (multituberculate-therian) clade is adopted here (Fig. 3E: node 4).

1.2.4 Spalacotheroids

Spalacotheroids are basal therian relatives of the Mesozoic to Recent marsupials and placentals. The marsupial-placental clade, also known as crown Theria, is successively nested within the northern tribosphenidan mammals, the pretribospheric mammals, and spalacotheroids (Luo *et al.* 2001a; Martin and Rauhut 2005). Spalacotheroids provide evidence for inferring the ancestral conditions from which marsupials and placentals have evolved (Hu *et al.* 1997; Li and Luo 2006; Ji *et al.* 2009).

The spalacotheroid *Maotherium* has an ossified Meckel's cartilage, which is solidly held by the Meckel's groove (Fig. 3F) (Ji *et al.* 2009). The preserved part of Meckel's element is identical to the ossified Meckel's cartilage preserved in *Gobiconodon* (Li *et al.* 2003), and *Yanoconodon* (Luo *et al.* 2007a), but more gracile than in *Repenomamus* (Wang *et al.* 2001; Meng *et al.* 2003). Because the ossified Meckel's cartilage is connected to the ectotympanic and the malleus in *Yanoconodon,* and because *Maotherium* is similar to *Yanoconodon* in the Meckel's cartilage, it is inferred that *Maotherium* has a similar, ossified connection between the mandible and the middle ear (Fig. 3D).

The spalacotheroid *Zhangheotherium* has an ossified Meckel's cartilage, which was displaced, *post mortem,* from its original anatomical position (Meng *et al.* 2003). *Maotherium* helps to show that the Meckel's cartilage is connected to the mandible in spalacotheroids, reinforcing the observation on *Zhangheotherium.*

In the fossil of *Maotherium*, the posterior part of the Meckel's cartilage becomes separated medially from the mandible, while its anterior part was nestled in the Meckel's groove on the mandible, made possible by the mid-length curvature of Meckel's cartilage (Ji *et al.* 2009). This is similar to the condition of the eutriconodont *Yanoconodon*, and the embryonic monotremes. *Maotherium* and *Yanoconodon* both corroborate that, prior to its disconnection from the mandible by resorption of the Meckel's cartilage, the ancestral middle ear was already medially displaced by some distance from the mandible (Fig. 2).

1.3 Phylogenetic homoplasies in definitive mammalian middle ear

The three extant mammalian groups have all achieved full separation of the middle ear from the mandible. Eutriconodonts, multituberculates and spalacotheroids are nested inside the monophyletic clade of Mammalia, but these three fossil groups have different characteristics of the middle ear. Taken together, the distribution of middle ear characters among these groups represents a conspicuous case of homoplasy. Either the definitive mammalian middle ear is independently acquired in mammalian evolution three different times and then secondarily reversed in *Maotherium*, or the *Yanoconodon* and *Maotherium* have undergone two independent evolutionary reversals to re-connect the middle ear to the mandible from a mammaliaform ancestor that had already achieved a full and definitive mammalian middle ear.

Hypothesis A (Fig. 3: Hypothesis A): DMME was absent in the common ancestor of monotremes, eutriconodonts and the living therians, but DMME evolved separately in extant monotremes, and then in living therians. This gains support from the study showing that the fossil monotreme *Teinolophos* retained the cynodont-like ancestral condition of the postdentary trough for the mandibular middle ear (MMEC) (Rich *et al.* 2005), and from the evidence of this trough in the basal australosphenidans (Luo *et al.* 2001a, 2002; Martin and Rauhut 2005), in which the monotremes are nested (Luo *et al.* 2001a; Martin and Rauhut 2005; Rougier *et al.* 2007). This hypothesis is ambiguous with regard to *Hadrocodium* in that the separation of middle ear would have occurred separately in *Hadrocodium* (Fig. 3B) from extant monotremes (Fig. 2C), but not

in the Cretaceous fossil monotremes that retain the postdentary trough, as argued by Rich *et al.* (2005), and in monotreme-related australosphenidans (e.g., Martin and Rauhut 2005; Rougier *et al.* 2007). The clade of multituberculates and extant therians (Fig. 3: node 4) has achieved the DMME but only to have it reversed again in *Maotherium* that is nested inside the multituberculate-therian clade.

Hypothesis B (Fig. 3: Hypothesis B): The full separation was already accomplished in the common ancestors of *Hadrocodium* and the extant Mammalia (Fig. 3: nodes 2 and 3). If we assume DMME is ancestral to both *Hadrocodium* and Mammalia, then this mammalian condition must have been reversed twice: first in eutriconodonts, and then again in spalacotheroids. This hypothesis is in conspicuous conflict with the fact that in Cretaceous fossil monotremes and their putative australosphenidan relatives, the middle ear was most likely still attached to the mandible (Rich *et al.* 2005; Martin and Rauhut 2005; Rougier *et al.* 2007).

Both hypotheses encounter some difficulties because neither is in full congruence with all of the current fossil records, largely thanks to the conflicting characters in several fossils. But no matter which hypothesis one would prefer, it is abundantly clear that there have been multiple acquisitions of DMME, coupled with evolutionary reversals from the DMME that must have happened multiple times, in the fully resolved evolutionary tree mandated by the parsimony of all characteristics in addition to ear characters.

2. Patterns in Ontogeny

2.1 Embryogenesis in extant mammals

In the ontogeny of extant mammals, the embryonic precursors to the malleus and incus are differentiated from the posterior end of the Meckel's cartilage; the middle ear bones including the ectotympanic are connected to the mandible by the Meckel's cartilage of the first branchial arch (Fig. 2) (Gaupp 1913; Kuhn 1971; Zeller 1987, 1989; Maier 1993; Evans 1993; Sanchez-Villagra *et al.* 2002). In later stages of development, the middle ear bones are separated from

the mandible, via four morphogenetic events or processes: First, the anlagen of the ear bones are medially displaced from the mandible in monotremes (Fig. 2F, G) and in the majority of placental mammals (Zeller 1987), although not in marsupials (Maier 1993). The medio-lateral displacement of the middle ear from the mandible in early embryos is a primitive pattern of extant mammals, and the lack of this displacement is a derived character for marsupials (Maier 1993). Second, the middle ear shows an antero-posterior displacement, away from the jaw joint. As pointed out by Rowe (1996), this is critical for removing the middle ear from the mandible and from the temporomandibular joint. Third, the middle ear shows negative ontogenetic allometry relative to the size of the mandible and the cranium (especially its braincase) (Rowe 1996). In early embryonic stages, the middle ear bones are large relative to the mandible (Fig. 2B, G), and they are ossified early, terminating their growth much earlier than other skull structures. With their size fixed upon ossification, during the subsequent development they show negative allometry to the growth of the mandible and the cranium, both of which continue to increase in size, long after the middle ear is ossified. Therefore, the timing of ossification of the ear bones is critical for achieving this negative ontogenetic allometry, and is fundamental to the normal formation of the definitive mammalian middle ear. It follows that, if this ontogenetic allometry is disrupted, the middle ear may not be capable of separating from the mandible. Fourth, the Meckel's cartilage must be resorbed so that the middle ear can be physically disconnected from the mandible. The normal development (including the resorption) of the Meckel's cartilage is patterned by a network of genes that control the development of vertebrate skull structures (Kuratani 2004; Chai and Maxson 2006). Changes in gene patterning and developmental timing can alter the morphogenesis of the middle ear, and resulting in such evolutionary changes as the paedomorphosis of the Meckel's cartilage and the middle ear (Fig. 2).

2.2 Gene patterning of Meckel's cartilage and middle ear

Meckel's cartilage derives from cranial neural

crest cells; it serves as scaffolding for development of mandibular and middle ear elements. Normal development of the Meckel's cartilage including its eventual resorption, and embryogenesis of its derivatives, such as middle ear bones, requires a wide range of structural and homeobox genes. Genes expressed in development of the middle ear structure include *Hoxa2, Emx2, Sox9, Bapx1*, *Gsc, Type II Collagen* (Rhodes *et al.* 2003; Santagati *et al.* 2005; Amin and Tucker 2006). Morphogenesis of Meckel's cartilage in mammals also requires a variety of growth factors. This complex signaling network includes: *Transforming Growth Factors–ß* (*Tgf-ß*) and its receptor (Ito *et al.* 2002; Oka *et al.* 2007), *Connective Tissue Growth Factors* (*Ctgf*) (Ivkovic *et al.* 2003), *Fibroblast Growth Factor* (*Fgf*) (Tucker *et al.* 2004), *Epidermal Growth Factor* (*Egf*) (Shum *et al.* 1993), and *Bone Morphogenetic Proteins* genes (*Bmp*), among a list of other genes (Choi *et al.* 2007).

The Meckel's cartilage and its associated ectotympanic ring and the anterior (goniale) element of the malleus, which are crucial for the normal development of the mammalian middle ear, are known to be patterned by at least two signaling pathways. The first was *Fgf8-Bmp4* pathway and *Bapx1* gene, which help to mediate the ossification of the ectotympanic ring and the anterior (goniale) element, and influence the developmental variability of the ectotympanic and the malleus (Tucker *et al.* 2004). The most convincing study on gene patterning of the Meckel's cartilage is on the large gene family of *Transforming Growth Factor Beta* (*Tgf-ß*) (Oka *et al.* 2007). The Meckel's cartilage and the surrounding structure are patterned by *Tgf-ß* (upstream) to *Ctgf* (downstream) pathway and *Msx1* gene, mutation of which can cause a premature ossification of Meckel's cartilage in embryogenesis, resulting in permanent connection of the middle ear to the mandible. In normal chondrogenesis of Meckel's cartilage of the placental mammal *Mus* (wild type), the *Tgf-ß* to *Ctgf* signaling pathway controls the cellular proliferation; it stimulates the proliferation, and inhibits the terminal differentiation of chondrocytes in the Meckel's cartilage (Ito *et al.* 2002; Ivkovic *et al.* 2003; Oka *et al.* 2007). It was demonstrated that mutant

Tgfbr2 fl/fl;Wnt1-Cre genes (mutant of *Tgf-ß*) accelerate chondrocyte proliferation and cause ossification of Meckel's cartilage in mutant *Mus* (Oka *et al.* 2007).

The phenotype of ossified Meckel's cartilage in *Tgfbr2 fl/fl;Wnt1-Cre* mutant mice is very similar to the prematurely ossified Meckel's cartilage fossilized in eutriconodonts (Fig. 2) and the spalacotheroid *Maotherium* (Fig. 3) (Luo *et al.* 2007a; Ji *et al.* 2009). This suggests that some similar developmental pathway, such as *Tgf-ß*-mediated and/or *Ctgf*-mediated control of chondrocyte proliferation had impacted the morphogenesis of the middle ear in Mesozoic mammals, such as eutriconodonts and spalacotheroids.

The genes for morphogenesis of the definitive mammalian middle ear and the Meckel's cartilage are also known for morphogenesis of the mandible in birds and even in zebra fish (Tucker *et al.* 2004; Eames and Schneider 2008). The signaling pathway and regulatory gene network of the branchial arches, such as the *Fibroblast Growth Factor* (*Fgf*) and *Bone Morphogenetic Proteins* (*Bmp*) are ancestral for all vertebrates with jaws (Kuratani 2004; Chai and Maxson 2006). The distinctive paedomorphic pattern of Meckel's cartilage in eutriconodonts and spalacotheroids can be attributed to the genetic regulatory network that has a much wider distribution in vertebrate phylogeny, outside mammals. The genes and pathways for the mammalian middle ear development belong to the "plug-in" subcircuits in hierarchical developmental Gene Regulatory Networks (dGRN) that are repetitively re-deployed throughout metazoan evolution, serving as a mechanism for evolutionary novelties including homoplasies in the mammalian middle ears (more discussion below) (Davidson and Erwin 2006; Erwin and Davidson 2009).

The knockout genetic experiments are sufficient to demonstrate that development of given phenotypic characters, such as those which can be fossilized in Mesozoic mammals, would require the normal function of a gene. However, it should also be pointed out that all developmental genes

are pleiotropic and their mutants would have a cascade of abnormal and ectopic structures, which are neither in fossil records, nor in the normal development of extant mammals (Smith and Schneider 1998). The mutation of individual developmental genes, no matter how powerful, does not represent a recapitulation or atavistic reversal in evolution. Rather, it can show in a general way which genes are involved in patterning the extant mammals, and that the gene patterning can be extrapolated to interpret phylogenetic evolution as documented by fossils.

3. Integrating Development into Phylogeny

Transformation from the pre-mammalian mandibular middle ear to the definitive mammalian middle ear is well documented by numerous fossils. On a broad scale of synapsid evolution, transformation of the mandibular ear in pre-mammalian synapsids appears to be an example of orderly evolution of a major mammalian apomorphy, shedding light on how a complex structure was assembled in phylogeny (Crompton 1972; Allin 1975; Luo 1994; Sidor 2001).

However, new and well-preserved fossils from the last 10 years, since the discoveries of the euticonodont *Repenomamus* and the mammaliaform *Hadrocodium* (Li *et al.* 2000; Luo *et al.* 2001b), have shown clearly that the detachment of the middle ear from the mandible is homoplastic within crown mammals. This assessment is fundamentally based on a robust phylogeny of all mammals (Fig. 3). By parsimony of all osteological and dental characteristics of Mesozoic and extant mammals (Luo and Wible 2005; Luo *et al.* 2007b), *Maotherium* and its spalacotheroid clade are more closely related to living therians, than multituberculates and eutriconodonts (Fig. 3: node 5). Eutriconodonts, including both gobiconodontids and *Yanoconodon* are placed between extant monotremes and the multituberculate-therian clade (Fig. 3: node 4). The middle ear features in these fossil groups have multiple acquisitions of derived conditions, or multiple reversals, when mapped on a fully resolved evolutionary tree.

The labile evolution of these middle ear char-

acteristics may well have been facilitated by labile developmental patterns. Paedomorphosis, or retention of fetal or juvenile characteristics of ancestors or phylogenetic relatives through developmental heterochrony, is a common phenomenon in vertebrate evolution. The ossified Meckel's cartilage connecting the middle ear to the mandible in eutriconodonts and spalacotheroids, and the relatively larger middle ear bones of *Yanoconodon,* bear strong paedomorphic similarity of the embryonic middle ear and Meckel's cartilage of extant monotreme and placental embryos (Fig. 2). The premature ossification of Meckel's cartilage in *Yanoconodon* and *Maotherium* represents a simple change in timing in ontogeny, or heterochrony, by comparison to extant mammals.

The genes and pathways for mammalian middle ear development all belong to the subcircuit rank in hierarchical developmental Gene Regulatory Networks (GRN), whose changes can impact evolution (Davidson and Erwin 2006; Erwin and Davidson 2009). Davidson and Erwin (2006) postulate that many signaling pathways and homeobox genes are the "plug-in" subcircuits, a fundamental part in the architecture of the Gene Regulatory Networks of all eumetazoans including mammals. The "plug-in" subcircuits have been re-deployed throughout the evolution of eumetazoan animals, and in different lineages. I would hypothesize that these ancient eumetazoan subcircuits, such as *Tgf-ß*-to-*Ctgf* pathway and *Hoxa2,* are repetitively co-opted into the ossification of the Meckel's cartilage and to cause the arrest of the negative ontogenetic allometry typical of extant mammals. This is likely a key developmental mechanism for the homoplastic evolution in the Meckel's cartilage and the mammalian middle ear. However, it is not well understood why these were activated only for some lineages, but not in others.

From the morphological standpoint, the mechanism of retaining the middle ear's connection to the mandible is simple. The middle ear of *Maotherium* and eutriconodonts shows a paedomorphic resemblance to the embryos of modern monotremes and placentals in which the middle ear is medio-laterally displaced from, but

still connected via Meckel's cartilage to the mandible (Fig. 2). All that is necessary for adult eutriconodonts and spalacotheroids to retain this paedomorphic mandibular ear connection is a relatively earlier timing in ("premature") ossification of the Meckel's cartilage. The resorption of Meckel's cartilage, as seen in the embryonic or fetal stages of extant mammals, simply did not happen due to a change that is not unlike the altered *Tgf*-β *to Ctgf* pathway (Oka *et al*. 2007) or *Fgf5-Bmp4* pathway (Tucker *et al*. 2004). And the middle ear bones must have also ossified relatively late, relative to the developmental schedule of the mandible, so these bones never had the negative ontogenetic allometry or the early ossification and size fixation. The homoplastic separation of the middle ear from the mandible in Mesozoic mammals is seemingly complex when mapped on a well-resolved phylogenetic tree (Fig. 3), but it is correlated with ontogenetic heterochrony and the developmental changes in gene patterning, both of which are universal in vertebrate development. Extrapolating these common and well-corroborated developmental patterns of extant mammals into the fossil record can illuminate the driving mechanism for a similar evolution to occur multiple times.

Reciprocally, fossil records can provide an insight for the phylogenetic scope of developmental homoplasies. In the case of the definitive mammalian middle ear, the labile phase of evolution with multiple reversals to "reconnect" the middle ear to the mandible via ossified Meckel's cartilage is only found in eutriconodonts and spalacotheroids, both basal in the mammal phylogenetic tree. No example has been known after the rise of the more derived extant marsupials and placentals and their immediate relatives, presumably because the evolution of their coiled cochlea in the inner ear in advanced therians (Ruf *et al*. 2009) may have ended the labile evolution of the mammalian middle ear (Martin and Ruf 2009). Among living mammals, an ossified Meckel's cartilage occurs only in certain mutant mice; the cartilage is retained only in pathological cases among humans. However, ossified Meckel's cartilage evolved at least twice in Mesozoic spalacotheroids and eutriconodonts. The absence of ossified Meckel's cartilage in the adult in extant monotremes, marsupials and placentals represents a more canalized development of the middle ear for these living lineages, in contrast to a much more labile evolutionary development of the middle ear features, underlined by a Gene Regulatory Network inherent in development of all eumetazoans including mammals, made possible by a greater diversity of about 20 Mesozoic mammaliaform clades for a vast range of evolutionary experimentations (Kielan-Jaworowska *et al*. 2004; Luo 2007). Development impacted phylogenetic evolution, but only within certain phylogenetic constraint.

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