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Review

Embryological and genetic aspects of middle ear development

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The process of hearing involves the reception and transmission of sound waves to the neural receptors, and their conversion into nervous impulses that can be processed by the corresponding areas in the central nervous system. This process is performed by the hearing apparatus that in mammals consists of three compartments, the external, middle and inner ears. Aerial sound is conducted through the external ear toward the tympanic membrane (or eardrum). Vibrations produced in this membrane are transmitted into the inner ear through a chain of three small ossicles located in the middle ear. In the inner ear specialized receptors translate vibrational energy into nervous information which reaches the brain through the eight cranial nerve.

The middle ear not only transmits vibrations from the eardrum into the inner ear. It also amplifies this signal so that relatively weak vibrational energy from the air can overcome the high inertia of the inner ear fluids. Furthermore, the morphological characteristics of the middle ear (i.e., volume of the cavities, ossicle mass and stiffness, etc.) determine the frequency of the sounds suitable to be transmitted (Moore, 1981).

Clinical experience has provided uncountable examples to show that the integrity of the conductive apparatus, represented by the external and middle ears, is essential for proper hearing. It has also been suggested that the integrity of the peripheral region of the auditory system is essential for the proper maturation of more central areas involved in hearing (Saunders *et al.*, 1993).

In many species (e.g., mouse or rat) the middle ear is not a completely functional organ at birth and must undergo a considerable degree of maturation during the first period of extrauterine life. However, most of the morphogenesis of the anatomical components of the middle ear occurs during intrauterine development. It is in the elucidation of the cellular and molecular basis of those morphogenetic processes where the most remarkable advances have been made in recent years. The availability of new techniques, in particular the possibility to create mouse strains carrying specific mutations (Capecchi, 1989), has resulted in the identification of several genes essential for the morphogenesis of different components of the

Abbreviations used in this paper: ET-1, Endothelin-1; EAM, external acoustic meatus; gsc, goosecoid; RA, retinoic acid; RAR, retinoic acid receptor; r, rhombomere.

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middle ear. In this review, I will focus on the intrauterine phase of middle ear development with special emphasis in the experimental data accumulated in the last years.

Preliminary considerations

In order to understand the development of the middle ear, some anatomical and evolutionary aspects should be clarified.

Anatomical considerations

Details in the middle ear anatomy vary among the different mammalian species and, therefore, only a general description will be outlined here. I will consider as the middle ear the structures located between the tympanic membrane, laterally, and the inner ear, medially (Fig. 1). It is composed of a cavity that contains the ossicle chain and several nerves, muscles and blood vessels.

In the adult animal, the middle ear cavity is enclosed in the temporal bone. This cavity is composed of two different parts: a main chamber, the tympanic cavity, where the ossicles are located, which connects with the pharynx through the pharyngotympanic tube; and the epitympanic recess, located above the tympanic cavity.

The tympanic membrane is located in the lateral wall of the tympanic cavity and separates the middle ear from the external acoustic meatus. It is composed of three layers: a fibrous stratum that gives elasticity to the eardrum; and two epithelia that cover laterally and medially the fibrous stratum, which are provided by the external acoustic meatus and the tympanic cavity respectively (Carlson, 1994). During fetal life, the tympanic membrane is supported on a semiannular bone, the tympanic ring, that later becomes incorporated into the temporal bone (Novacek, 1993).

The ossicle chain connects the tympanic membrane with the inner ear. From lateral to medial we find the malleus, incus and stapes. The malleus has four parts: a manubrium, which is inserted in the fibrous stratum of the eardrum and provides the physical connection of the ossicle chain to the tympanic membrane; a head, which articulates with the incus; a neck that connects the manubrium and head of the ossicle; and a *processus brevis* protruding from the connection between the manubrium and the neck. The incus consists of a head that articulates with the malleus, a short process, and a long process that connects with the stapes. The stapes connects the ossicle chain with the inner ear by the insertion of its footplate in the oval window of the vestibule. This ossicle also contains an arch that articulates with the incus.

Two muscles have functional relevance in the middle ear: the tensor tympani, inserted medially in the cartilaginous part of the pharyngotympanic tube and laterally in the manubrium of the malleus; and the stapedius, inserted in the roof of the middle ear cavity and in the stapedial arch, close to the incudo-stapedial joint. Finally, a number of nerves and blood vessels are involved in the innervation and vascular supply of the different components of the middle ear. Of these, only two have major anatomical relations in the area: the chorda tympani, a branch of the facial nerve that crosses the middle ear without functional contribution to the area; and in some mammals the stapedial artery, which crosses the stapedial foramen (delimited by the arch and the footplate of the stapes).

Evolutionary considerations

The middle ear is an exclusive feature of mammals. Extensive studies have established that the ossicle chain is the phylogenetic equivalent of the jaw joint in all non-mammalian jawed vertebrates, typically exemplified by the reptiles (Novacek, 1993) (Fig. 2). In these animals, the jaw joint is established between the quadrate portion of the palatoquadrate, above, and the articular, derived from the caudal region of Meckel's cartilage, below. In the typical living reptile, sound is transmitted through the *columella auris* from a primitive tympanic membrane to the inner ear (Rieppel, 1993). During therapsid evolution, the jaw joint was modified, possibly in connection with the evolution of the neocortex (Rowe, 1996), and became established between the dentary and the squamosal bones. Concomitantly, the articulo-quadrate joint evolved to constitute the mammalian middle ear. The articular became the malleus and the quadrate the incus, and these ossicles, through their respective contacts with the tympanic membrane and stapes [the phylogenetic homolog of the stapedial portion of the columella (Novacek, 1993)] were incorporated into the sound-transmitting apparatus (Hopson, 1966; Allin, 1975). The mammalian tympanic membrane is also a neoform (Presley, 1984). As discussed above, it is supported in the tympanic ring, which is the homolog of the reptilian angular bone (Novacek, 1993).

Embryological origin and morphogenesis of the middle ear

Ossicle chain and tympanic ring

The skeletal elements of the middle ear develop from the mesenchyme of the first two branchial arches (Fig. 3) (Carlson, 1994). Although slightly different hypotheses have been formulated (Cauldwell and Anson, 1942; McPhee and van der Water, 1988), in general terms, it can be considered that the malleus, incus and tympanic ring are all first arch derivatives, while the stapes derives from the second branchial arch (Carlson, 1994).

The malleus and incus are formed mainly by endochondral ossification. Only the malleal anterior process, which is added later in development to the core of the ossicle, undergoes endomembranous ossification as the gonial bone (Novacek, 1993). The development of the malleus and incus starts as a condensation of concentric cells in the caudal extremity of Meckel's cartilage (Miyake *et al.*, 1996). This extends perpendicular to the main axis of the Meckel's cartilage toward the otic capsule. This condensation then separates into two parallel components that remain connected dorsally but separate ventrally (Miyake *et al.*, 1996). The most caudally located of these components will form the incus, and the most rostral the malleus, which remains attached to the Meckel's cartilage for a long time. In some species, like the mouse, it only separates after birth. In the mouse, the first sign of cartilage differentiation appears in these condensations about one day later, first with the deposition of sulphated proteoglycans and then shortly afterward with the synthesis of type II collagen (Miyake *et al.*, 1996).

Experiments involving time-controlled retinoic acid (RA) treatments suggested that the malleus is formed from at least two condensation centers, located in the head and manubrium (Mallo, 1997). These experiments did not differentiate whether the neck of the malleus is formed from an independent condensation center or from the extension of either one or both of the others. Genetic data indicate that a normal neck can coexist with a seriously affected manubrium (Martin *et al.*, 1995; Rivera-Pérez *et al.*, 1995; Yamada *et al.*, 1995), suggesting that the contribution of the center located in the manubrium may not be very important.

Unlike the malleus and incus, the tympanic ring is formed by endomembranous ossification. It starts as a single condensation in the region of the first branchial arch, near the caudal extremity of Meckel's cartilage (Mallo and Gridley, 1996). This condensation grows ventrally in an annular fashion and finishes in the area of the second branchial arch (Mallo and Gridley, 1996). The differentiation of this initial condensation has a similar temporo-spatial pattern. In the mouse, the first signs of osteoid deposition can be detected by late day 14 post-fertilization, and it is complete roughly 2 days later (Mallo and Gridley, 1996).

The origin of the stapes is generally considered to be in the second branchial arch (Carlson, 1994). However, a mixed origin of this ossicle has also been suggested (Takeda *et al.*, 1996). In particular, some investigators consider that some parts of the stapedial footplate derive from the otic capsule (Cauldwell and Anson, 1942). Whatever its precise origin, it is clear that this ossicle is formed by endochondral ossification. Evidence from mouse and human embryos suggests that the stapes develops from two separate primordia, one for the footplate, probably connected to the otic capsule, and one for the arch (Hanson *et al.*, 1962; Mallo, 1997). The latter develops from the dorsal part of the second arch in close connection with the stapedial artery which it finally surrounds (Anson and Cauldwell, 1942). It seems possible that the correct development of each of the parts of the stapes occurs to a large extent independently of the other. This inference is supported by a variety of abnormalities in the mouse resulting from the administration of different teratogens (Louryan and Glineur, 1992; Takeda *et al.*, 1996; Mallo, 1997) and from the inactivation of particular genes (Martin *et al.*, 1995; Qiu *et al.*, 1995,1997) in which, either a stapedial footplate or arch is apparently perfectly formed in total or partial absence of the other. Furthermore, it is clear that the morphogenesis of the stapes occurs independently of any interaction with the incus, since normal stapes can be found in animals lacking the incudal long process (Mallo, 1997).

Embryologically, the skeletal components of the middle ear derive from the cranial neural crest (Le Douarin *et al.*, 1993). These are cells that delaminate from the dorsal part of the developing neural tube and migrate into the branchial arches and frontonasal mass where they contribute to a wide variety of tissues, including all the bones and cartilages of the face (Le Douarin *et al.*, 1993). Relevant to our discussion are the neural crest cells that migrate from two regions of the developing brain: the caudal midbrain and the rostral hindbrain (Fig. 3). At this point it should also be mentioned that the developing hindbrain is divided into a series of segments called rhombomeres (r), that represent developmental compartments (Lumsden and Keynes, 1989).

That the middle ear elements are of neural crest origin, and the precise region of the neural tube from which the neural crest contributing to each of the elements migrates, has been established mainly by the extrapolation of avian data based on phylogenetic homologies (Novacek, 1993). Detailed studies were possible in avian embryos because of their accessibility to embryonic manipulations including different types of graftings (Noden, 1984), which are not possible in mammalian embryos. However, even if we cannot be completely sure about the accuracy of this evolutionary extrapolation, particularly in the details, some experimental data suggest that it may be, to a large extent, valid. Experiments in mice have traced a variety of ear malformations to

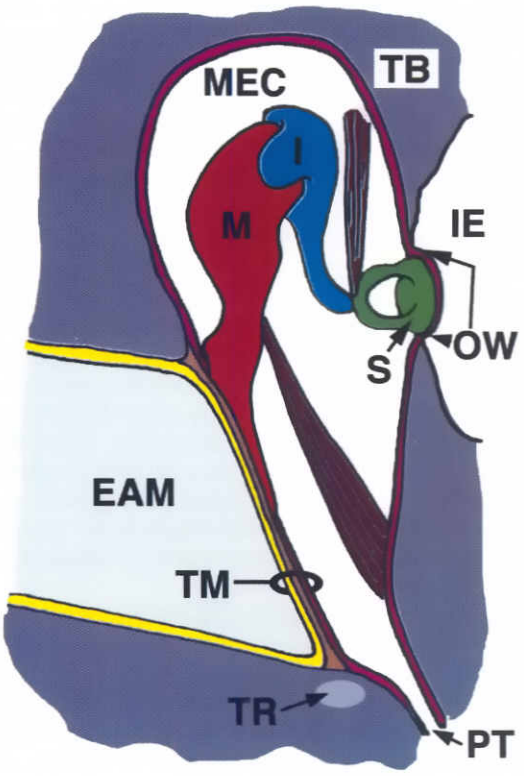

Fig. 1. Schematic representation of the middle ear. *It is located in the temporal bone (TB) between the external acoustic meatus (EAM), which connects with the open air, and the oval window (OW) located in the inner ear (IE). The malleus (M) is inserted in the tympanic membrane (TM), which has three layers: an external epithelium (yellow) provided by the EAM, a middle fibrous layer (brown) and an internal epithelium (pink) provided by the middle ear cavity (MEC). The tympanic membrane is supported on the tympanic ring (TR). The malleus connects with the incus (I) and the latter with the stapes (S), inserted in the oval window. The ossicles are located in the middle ear cavity which connects with the pharynx through the pharyngotympanic tube (PT).*

a defective behavior of the neural crest (Webster *et al.*, 1986; Pratt *et al.*, 1987; Mallo, 1997), indicating that those cells contribute to the formation of the skeletal elements of the ear. Also, a comparison of the migration of the neural crest in chicken and rodent embryos has shown that they behave very similarly (Lumsden *et al.*, 1991; Serbedzija *et al.*, 1992; Osumi-Yamashita *et al.*, 1994). For instance, the different branchial arches are populated by neural crest cells arising from the same rostrocaudal areas of the developing brain. Moreover, the skeletal elements considered as evolutionary homologs develop from similar regions in avian and mammalian embryos (Carlson, 1994; Köntges and Lumsden, 1996). Finally, the inactivation of *Hoxa-2* (Gendron-Maguire *et al.*, 1993; Rijli *et al.*, 1993), which is expressed in the neural crest of the second branchial arch (Prince and Lumsden, 1994; Mallo, 1997), produced a phenotype in the skeletal elements of the middle ear phylogenetically similar to that resulting from heterotopic grafts in chicken (Noden, 1983a) that might have also induced a down regulation of *Hoxa-2* in the second branchial arch (Prince and Lumsden, 1994).

Therefore, the discussion about the origin of the skeletal elements of the middle ear will be mainly based on the detailed studies of the origin of their avian homologs (Couly *et al.*, 1996; Köntges and Lumsden, 1996). According to the latter, the three first branchial arch elements derive from different combinations of neural crest cells migrating from the caudal midbrain and the first two rhombomeres (Fig. 3). The tympanic ring would develop from midbrain and r1 neural crest, the malleus would derive from midbrain and r1 and r2 neural crest and the incus would be formed from r1 and r2 crest cells. The second branchial arch element of the middle ear, the stapes, would derive from rhombomere 4 (Fig. 3). As discussed above, part of this ossicle might originate from the otic capsule (Cauldwell and Anson, 1942) suggesting its mesodermal origin. However, tracing experiments in birds have shown that part of the area of the otic capsule where the oval window is located is also derived from the neural crest (Couly *et al.*, 1993). Therefore, even if it develops as part of the otic capsule, the stapedial footplate might still be of neural crest origin. This possibility is also supported by experiments in which the interference with the neural crest at particular stages resulted in the failure of the stapedial footplate to develop (Mallo, 1997).

The timing of the migration of the neural crest cells that will form each of the different middle ear elements follows a typical sequence that in mouse occurs between day 8 plus 4.5 h and day 8 plus 7.5 h post-fertilization (Mallo, 1997). This was determined by the analysis of the phenotypes resulting from the blockage of neural crest migration at slightly different time points of mouse development. According to those experiments the first crest cells to start migration are those contributing to the head of the malleus, followed by those forming the head of the incus. The next to migrate are r4 crest cells contributing to the stapedial footplate. The neural crest cells that make the tympanic ring and the manubrium of the malleus start their migrations shortly thereafter. The coincidence in the migration time of these last two elements is interesting considering that both are involved in the formation of the eardrum; it might reflect a tightly coordinated mechanism in the morphogenesis of the elements that form the tympanic membrane. However, it should be noted that mechanisms other than the timing of neural crest migration might be involved in the coordination of this developmental program (see later). The crest cells involved in the formation of the stapedial arch start their migration afterwards, followed by those contributing to the neck of the malleus. The last cells to migrate are those that form the long process of the incus. Of course, this description is somewhat artificial and focuses on the sequence of the initiation of the migration of the neural crest contributing to each of the elements. However, it should be pointed out that there is substantial overlap in the migration times so that when the cells that contribute to one element start to migrate, neural crest cells forming other elements whose development had already started continue to migrate until their morphogenesis is complete.

Muscles

There are no detailed analyses of the embryological origins of the muscles of the middle ear. However, if they follow the same patterns as the other muscles in the craniofacial area (Noden, 1983b), they develop from the paraxial mesoderm that migrates into the branchial arches.

Recent data from birds (Köntges and Lumsden, 1996) indicate that, even though the neural crest does not contribute to the formation of the musculature of this area, it patterns the muscular insertions in the skeleton. This has been suggested after the finding that the tissues at the insertional places are of neural crest origin. This might be just another example of a mechanism to coordinate the proper formation of complex structures derived from diverse origins.

Middle ear cavity

The middle ear cavity develops from the first pharyngeal pouch (Carlson, 1994). This is a lateral expansion of the developing pharynx located between the first and second branchial arches. The end of the pouch expands to form a primary middle ear cavity that maintains an communication with the pharynx through the pharyngotympanic tube (Carlson, 1994). This primary cavity reaches the region of the tympanic membrane, to which it provides the internal epithelium. However, it does not extend to the ossicles which, at this stage, remain encased in mesenchymal tissue (Novacek, 1993). The formation of a definitive tympanic cavity starts normally after birth, with the resorption of the mesenchymal tissue, the connection to the primary cavity and the formation of a dense fibrous tissue or cartilage that will comprise most or all of the walls of the definitive tympanic cavity (Novacek, 1993).

Indirect evidence in the mouse from different experimental systems indicate that the primary tympanic cavity might be initially formed in a quite passive way (Mallo, 1997). Once the first pharyngeal pouch is made morphogenetic movements of the structures around it (in particular the developing branchial arches) might result in the formation of an initial tubotympanic recess. Interference with the formation of the pharyngeal pouch (Goulding and Pratt, 1986; Webster *et al.*, 1986; Pratt *et al.*, 1987; Lee *et al.*, 1995; Mallo, 1997) results in the failure of the middle ear cavity to develop (Mallo, 1997). This first recess would be later expanded and restructured in association with the development of other structures in the area, particularly the tympanic membrane, to form the above mentioned primary tympanic cavity and pharyngotympanic tube.

The development of the epitympanic recess occurs independently of the main chamber. Detailed studies in humans (Tono *et al.*, 1996) revealed that it forms after birth, initially as a mesenchyme filled cavity separated from the tympanic cavity by a bony plate and a mucosal fold. This primary epitympanic recess then pneumatizes by resorption of the mesenchyme and opens a direct communication with the rest of the middle ear cavity.

Tympanic membrane

The tympanic membrane has a triple origin (Carlson, 1994). The outer epithelial cover is formed from the ectodermal epithelium of the first branchial cleft, located between the first and second branchial arches. The internal epithelium derives from the first pharyngeal pouch that expands to form the tympanic cavity. It is therefore endodermal in origin. The fibrous stratum originates from the mesenchyme of the branchial arches that becomes enclosed between the two epithelia when they approach and appose to each other.

The morphogenesis of the eardrum starts with the formation of the tympanic cavity and external acoustic meatus (EAM) which then come together to form a functional membrane. Studies in mouse and human embryos have shown that the EAM develops in close association with the tympanic ring (Declau *et al.*, 1989; Mallo and Gridley, 1996). The formation of the EAM (Fig. 4) starts as a

solid epithelial invagination of the first branchial cleft, frequently referred to as the meatal plug (Declau *et al.*, 1989; Michaels and Soucek, 1989; Nishimura and Kumoi, 1992; Mallo and Gridley, 1996). This epithelial invagination is first directed towards the initial condensation of the tympanic ring in the area of the first branchial arch (Michaels and Soucek, 1989; Mallo and Gridley, 1996). As the tympanic ring grows around the cleft into the region of the second branchial arch, the leading edge of the EAM follows it closely in a circular movement (Fig. 4) (Michaels and Soucek, 1989; Mallo and Gridley, 1996).Then, the medial surface of the meatal plug starts to flatten along the plane defined by the tympanic ring and to associate with the lateral epithelium of the tympanic cavity, entrapping the manubrium of the malleus in between (Michaels and Soucek, 1989; Mallo and Gridley, 1996). This process is initiated ventrally and extends dorsally to cover all the surface of the eardrum (Fig. 4) (Mallo and Gridley, 1996). In the mouse, the basic development of the tympanic membrane occurs between early day 13 and day 16 (Mallo and Gridley, 1996); in humans between the 7th and 18th weeks of gestation (Michaels and Soucek, 1989). A final step in the formation of the tympanic membrane involves the opening of the external acoustic meatus so that the eardrum comes in contact with the air (Michaels and Soucek, 1989).

Several experiments in mouse embryos suggest that the formation of the inner and outer layers of the eardrum are initially formed by different and independent mechanisms. The primordium for the inner layer is provided by the tympanic cavity which, as discussed above, is formed in a quite passive fashion (Mallo, 1997). Conversely the formation of the external surface, seems to be an active process induced and/or coordinated by the tympanic ring (Mallo and Gridley, 1996). Clinical experience from humans indicate that the absence of the EAM or its reduction in particular types of atresia correlate with problems in the formation of the tympanic ring (Lambert and Dodson, 1996). Furthermore, experimental deletions of the tympanic ring were in all the cases that have been analyzed associated with the absence of tympanic membranes (Rivera-Pérez *et al.*, 1995; Yamada *et al.*, 1995; Mallo and Gridley, 1996). In *goosecoid* (*gsc*) mutant mice, both the external acoustic meatus and the tympanic ring fail to form (Rivera-Pérez *et al.*, 1995; Yamada *et al.*, 1995). In these animals, however, a rather normal middle ear cavity was formed. *gsc* is expressed in the mesenchyme surrounding the first pharyngeal cleft (from which the tympanic ring develops) but not in the epithelial primordium of the EAM (Gaunt *et al.*, 1993; Mallo and Gridley, 1996). Since *gsc* is a transcription factor (Cho *et al.*, 1991) and thus expected to be cell autonomous, the absence of the EAM in the $gsc^{-/-}$ animals is very likely secondary to the lack of tympanic ring (Mallo and Gridley, 1996). Further support for this hypothesis comes from the results of total or partial deletion of the tympanic ring upon particular RA treatments of mouse embryos (Kessel, 1992; Mallo and Gridley, 1996; Mallo, 1997). In such fetuses the extent to which the EAM was formed was in direct correlation with the length of tympanic rings formed. In the most extreme cases, when the tympanic ring was completely absent, the external acoustic meatus failed to form (Mallo and Gridley, 1996; Mallo, 1997). A complementary situation was found in *Hoxa-2* null mutant mice. In those animals the tympanic ring is duplicated (Gendron-Maguire *et al.*, 1993; Rijli *et al.*, 1993). Analysis of the ear region of $Hoxa\text{-}2^{-/-}$ embryos revealed that the EAM was also duplicated, with each duplicate directed toward a tympanic ring to form a kind of double tympanic membrane (Mallo and Gridley, 1996). Again, the absence of *Hoxa-2* is expected to have a primary effect on the development of the supernumerary tympanic ring which would be, in turn, responsible for the formation of the extra EAM.

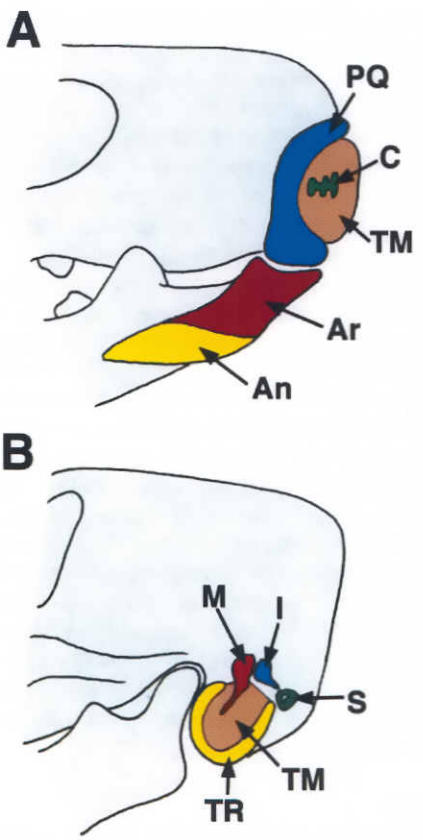

Fig. 2. Phylogenetic homologies between the reptilian jaw joint and the mammalian middle ear. **(A)** *In the reptiles the jaw joint is established between the palatoquadrate (PQ) and the articular (Ar), and the sound is transmitted from the tympanic membrane (TM) by the columella (C). (An) angular bone.* **(B)** *During evolution the mammalian jaw joint was established between the dentary and the temporal bones and the elements of the reptilian jaw joint evolved to make the middle ear. (I), incus; (M), malleus; (S), stapes; (TR), tympanic ring. The phylogenetic equivalents are indicated with the same color.*

The EAM seems to be formed by an active mechanism. The $gsc^{-/-}$ animals show no obvious defects in the formation of the branchial cleft, but the EAM fails to form (Rivera-Pérez *et al.*, 1995; Yamada *et al.*, 1995). Therefore the tympanic ring might exert some kind of active effect on the epithelium of the cleft to invaginate. The results of RA experiments in mouse embryos also support this view. The RA-induced loss of the first branchial cleft and pouch resulting from the fusion of the first and second branchial arches (Goulding and Pratt, 1986; Webster *et al.*, 1986; Pratt *et al.*, 1987; Lee *et al.*, 1995), was associated with the absence of middle ear cavity but not with the absence of the EAM if the tympanic ring was formed (Mallo and Gridley, 1996; Mallo, 1997). These experiments also showed that the morphogenesis of

the outer part of the tympanic membrane can go very far even without the contribution of the internal epithelium. When in those animals a complete tympanic ring was formed, it was accompanied by the formation of a largely normal looking lateral half of the tympanic membrane, with the EAM flattened in the plane of the ring in close contact with the manubrium of the malleus (Mallo, 1997). Only the endodermal part of the membrane seemed to be missing, likely because the fusion of the first two arches hampered the formation of the tympanic cavity (Mallo, 1997).

The mechanism by which the tympanic ring organizes the formation of the tympanic membrane is not clear. Of the different theoretical possibilities, the hypothesis of a physical pulling mechanism seems to be favored by existing data. The flattening of the EAM in the plane of the tympanic ring starts to be evident when the development of this bone has completed a considerable arch about its circumference and progresses from the middle of the arch toward the free ends (Michaels and Soucer, 1989; Mallo and Gridley, 1996). It is therefore possible that the tympanic ring secretes one or several extracellular matrix proteins that later become organized in a net, like cables extending from one part of the ring to another. These molecules would attach to the EAM, initially pulling its leading edge in and then attracting its medial surface to the plane of the ring. At the same time this process is occurring, the lateral epithelium of the tympanic cavity would also be dragged toward the same area, possibly by a similar mechanism, to form a functional eardrum.

Oval window

This structure is located in the lateral surface of the otic capsule, which is of mesodermal origin (Le Douarin *et al.*, 1993). However, as already discussed above, experiments in birds have suggested that at least part of the cartilage in the area where this window is located derives from the neural crest (Couly *et al.*, 1993). If this is the case, it could be hypothesized that it arises from neural crest derived from the fourth rhombomere because the oval window is lost in the *Hoxa-2* mutant mice along with the other second branchial arch structures (Gendron-Maguire *et al.*, 1993; Rijli *et al.*, 1993). However, even if in these mutant embryos the loss of the oval window is associated with the absence of the stapes (Gendron-Maguire *et al.*, 1993; Rijli *et al.*, 1993), the existence of this ossicle is not essential for the window to be formed (Louryan and Glineur, 1991; Mallo, 1997). The concomitant formation of the stapes might be, however, important for the proper formation of the fenestra because in the absence of the ossicle the window is always smaller and deformed (Louryan and Glineur, 1991; Mallo, 1997).

Genetic regulation of morphogenesis of the middle ear elements

In recent years there has been a major advance in knowledge about the genes involved in the correct morphogenesis of the middle ear. This information came basically from two sources: from the positional cloning of genes responsible for craniofacial diseases of genetic origin, and from the creation of mouse strains carrying mutations in potential regulatory genes expressed in the branchial area. The mechanisms by which such genes influence the development of the middle ear are diverse, owing to the complexities of the morphogenesis of this area. According to their role in the development of the middle ear, these genes can be grouped in three categories: a) genes involved in skeletal formation; b) genes involved in the development of the branchial arches; and c) genes involved in the development of specific areas of the middle ear.

Genes involved in skeletal formation

The malleus, incus, stapes and tympanic ring are part of the skeleton. Hence, genes implicated in bone and cartilage formation are also involved in their development. Several genes have already been identified as responsible, in mutant form, for human syndromes affecting skeletal development (Foster *et al.*, 1994; Reardon *et al.*, 1994; Wagner *et al.*, 1994; Putnam *et al.*, 1995; Bellus *et al.*, 1996; Sood *et al.*, 1996). In some of those syndromes the middle ear is also compromised. The most relevant to our discussion is *Sox9*, which has been shown to be responsible for campomelic dysplasia (Foster *et al.*, 1994; Wagner *et al.*, 1994). Patients carrying a variety of mutations in and around this gene show a variety of skeletal defects which include the middle ear elements (Foster *et al.*, 1994; Wright *et al.*, 1995). It has been shown that this gene directly regulates the expression of type II collagen (Bell *et al.*, 1997), the major structural component of cartilage, without which no endochondral bone is formed (Li *et al.*, 1995). Therefore *Sox9* is involved in the regulation of chondrogenesis (Graves, 1997) and thus the middle ear phenotype in campomelic dysplasia patients is likely the result of a more general effect of the mutation on cartilage formation.

It is important to point out that not all the mutations in genes that have a role in skeletal development show phenotypic effects in the ear region (Reardon *et al.*, 1994; Putnam *et al.*, 1995; Bellus *et al.*, 1996; Sood *et al.*, 1996). This might indicate that bones and cartilages in different areas of the body display different molecular characteristics, that redundancy is more effective in some skeletal elements than in others or that specific conditions of physical stress are required for a particular defect to be manifested.

Genes involved in the development of the branchial arches

As discussed above, the skeletal elements that derive from the branchial arches are of neural crest origin (Le Douarin *et al.*, 1993). However, for these cells to develop they must undergo a complex series of interactions with the epithelia of the branchial arches (Thesleff *et al.*, 1995). Therefore genes that are important for the behavior of the neural crest, and those encoding the signals provided by the arch epithelia, will have a role in the formation of the middle ear. In this section I will only consider those genes which have been shown to have broad effects on the development of the arch area. They include *Treacle*, *AP-2*, *Endothelin 1* (*ET-1*) and the receptors for retinoic acid (RARs) (probably reflecting a function for RA itself). Other genes involved in the development of specific elements of the middle ear will be considered in the next section.

The importance of *ET-1* in the development of the craniofacial region has been revealed from the consequences of its inactivation in mice (Kurihara *et al.*, 1994). Null mutants in the *ET-1* gene showed an almost complete absence of the skeletal derivatives of these two branchial arches, including the middle ear ossicles and tympanic ring, indicating that it plays a crucial role in the development of this area (Kurihara *et al.*, 1994). *ET-1* is a secreted peptide synthesized in the epithelia of the first and second branchial cleft

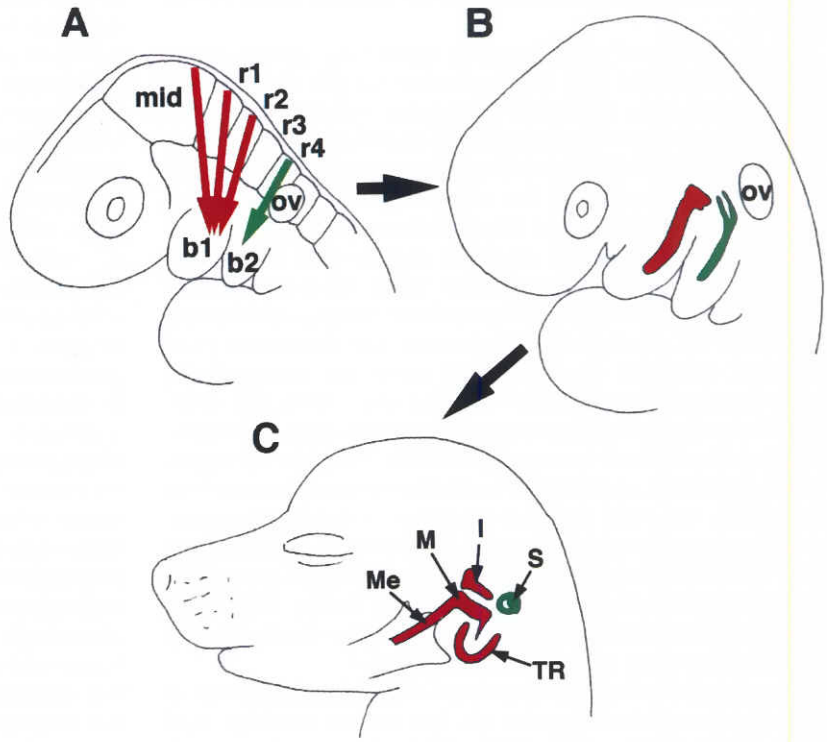

Fig. 3. Embryological origin of the middle ear skeletal elements. (A) *The bones and cartilages of the middle ear derive from the neural crest cells (indicated by colored arrows). Neural crest migrating from the midbrain (mid) and rhombomeres (r) 1 and 2 (red arrows) populate the first branchial arch (b1); the second branchial arch (b2) is populated by crest cells migrating from r4 (green arrow).* **(B)** *These neural crest cells form the primordia of the first (red) and second (green) branchial arch skeletal elements.* **(C)** *From the first branchial arch develop the malleus (M), attached to the Meckel's cartilage (Me), the incus (I), and the tympanic ring (TR). From the second branchial arch develops the stapes (S).*

early in development (Kurihara *et al.*, 1994). Additional analysis showed that receptors for *ET-1* are expressed in the arch mesenchyme (Barni *et al.*, 1995). Therefore, it is likely that *ET-1* is one of the factors provided by the epithelia of the branchial arches necessary for the development of the neural crest that had migrated into the branchial area. Whether this peptide has a general trophic effect on the neural crest cells, or directs their differentiation remains to be determined.

AP-2 also seems to have a general role in the development of the cranial neural crest. This gene encodes for a transcription factor expressed in the cells of the cranial neural crest (Mitchell *et al.*, 1991). Gene inactivation experiments indicated that *AP-2* plays a central role in the development of the craniofacial area (Schorle *et al.*, 1996; Zhang *et al.*, 1996). *AP-2*$^{-/-}$ mutants showed a striking failure in the development of the structures derived from the cranial neural crest (Schorle *et al.*, 1996; Zhang *et al.*, 1996). The affected structures included the middle ear. Further analysis of these mutants indicated that *AP-2* is not involved in the formation or migration of the crest cells (Schorle *et al.*, 1996; Zhang *et al.*, 1996). They rather suggested a role in the differentiation or survival of those cells in the branchial area. However, contrary to what has been discussed for *ET-1*, *AP-2* is likely involved in the behavior of the cranial neural crest itself because, as a transcription factor, it is expected to act cell autonomously in the cells of the neural crest.

Treacle has been recently cloned using a positional cloning approach, as it has mutant forms associated with the Treacher Collins syndrome (Treacher Collins Syndrome Collaborative Group, 1996). This is an autosomal dominant syndrome characterized by a variety of craniofacial malformations including defects in the external ears, malformation of the middle ear bones and atresia of the EAM (Phelps *et al.*, 1981). Given the extensive effects of mutations in *Treacle* on the development of the branchial area, it is thought that this gene is among the main players in the development of the neural crest that migrate into the branchial arches (Treacher Collins Syndrome Collaborative Group, 1996). It is classically considered that this syndrome results from a general defect in the formation, migration or differentiation of the cranial neural crest that populate the branchial region (Poswillo, 1975). However, so far it is unknown the stage in which *Treacle* is involved. Sequence analysis revealed no homologies with known genes and initial expression data showed a widespread distribution in the adult tissues (Treacher Collins Syndrome Collaborative Group, 1996). The elucidation of the role *Treacle* plays in the development of the branchial area awaits further analysis, including the study of its expression during the development of this region.

RA might also play a role in the morphogenesis of the middle ear, as revealed by the effects of joint inactivations of two of its receptors (Lohnes *et al.*, 1994). Those *RAR* mutations resulted in small malformations in the incus which contained a connection with the alisphenoid bone, and a complete absence of the stapes. Considering the broad effects of those mutations on the craniofacial area and the effects of the RA excess on premigratory neural crest (Morriss-Kay, 1993), it has been hypothesized that RA is required for the formation or migration of the cranial neural crest (Lohnes *et al.*, 1994). However, it is possible that not all the cranial neural crest cells require RA for their development because the malleus and tympanic ring remained unaffected by such RAR mutations (Lohnes *et al.*, 1994).

Genes involved in the development of specific areas of the middle ear

With the advent of gene targeting techniques several genes have been identified that are necessary for the formation of particular parts of the middle ear. This group includes *gsc, MHox, Hoxa-1, Hoxa-2, Dlx-1, Dlx-2* and *Msx-1* (summarized in Table 1).

The *Hox* genes were the first genes shown to have specific effects on the formation of the middle ear. Gene inactivation experiments have shown that *Hoxa-2* plays a central role in the development of the skeletal elements derived from the second branchial arch (Gendron-Maguire *et al.*, 1993; Rijli *et al.*, 1993). In its absence, the second arch structures fail to develop and instead, a duplicated set of proximal first branchial arch derivatives (malleus, incus, tympanic ring, squamous bone) are formed in the second branchial arch (Gendron-Maguire *et al.*, 1993; Rijli *et al.*, 1993). These duplicated elements adopt a mirror image configuration with respect to their normal counterparts. Thus, the ear region of these mutants is composed of a double malleus joined at their manubriums, two incus joined at the tips of their long processes and two incomplete tympanic rings. Furthermore, as discussed above, the external acoustic meatus is also duplicated generating an abnormal tympanic membrane (Mallo and Gridley, 1996). The stapes and the oval window are totally absent from these mutants (Gendron-Maguire *et al.*, 1993; Rijli *et al.*, 1993).

Gene expression studies have shown that *Hoxa-2* is expressed in the neural crest that migrates into the second branchial arch (Prince and Lumsden, 1994; Mallo, 1997). Conversely, the first branchial arch is completely devoid of *Hoxa-2* expression (Prince and Lumsden, 1994; Mallo, 1997). Therefore, this gene seems to alter the differentiation program of the mesenchymal second arch neural crest from a first arch default. *Hoxa-2* was initially proposed to act as a selector gene instructing the neural crest of the second branchial arch into a differentiation program leading to the formation of typical second arch structures (Gendron-Maguire *et al.*, 1993; Rijli *et al.*, 1993). More recently, however, it has been proposed that the action of *Hoxa-2* in the development of the second branchial arch is more indirect, modulating the way the second branchial arch crest cells respond to patterning signals provided by the arches' epithelia (Mallo and Brändlin, 1997). A final elucidation of the role of *Hoxa-2* in the patterning of the second branchial arch awaits the finding of its target genes.

Mutations in *Hoxa-1* have also resulted in defects in the middle ear, indicating that this gene might also play a central role in the formation of this area (Lufkin *et al.*, 1991; Chisaka *et al.*, 1992). However, the analysis of this role has been made difficult by the fact that the two available mutant strains show different phenotypes in the middle ear (Lufkin *et al.*, 1991; Chisaka *et al.*, 1992; Mark *et al.*, 1993). In one (Chisaka *et al.*, 1992) a complete deletion of the middle ear elements was reported while in the other (Lufkin *et al.*, 1991; Mark *et al.*, 1993) the middle ear phenotype was restricted to a minor defect in the insertion of the stapes in the oval window. It has been proposed that the different phenotypes derive from the inactivation of only one or the two transcripts that originate from this genomic region (Mark *et al.*, 1993). It is, however, still difficult to understand how the inactivation of *Hoxa-1* can affect the middle ear without being expressed in the cells from which it derives (Murphy and Hill, 1991). An intriguing possibility is suggested from recent findings showing that the RA-induced ectopic expression of *Hoxa-2* in the first branchial arch resulted in variable defects in the first arch ear elements (Mallo and Brändlin, 1997). As regulatory regions of *Hoxa-2* have been found in the genomic region of *Hoxa-1* (Frasch *et al.*, 1995), it is thus possible that the ear phenotypes in the *Hoxa-1* mutants are secondary to the alteration of *Hoxa-2* expression resulting from the gene targeting strategies. Analysis of *Hoxa-2* expression in these mutants is necessary to evaluate this possibility.

MHox is another homeobox containing gene that has proven to play a central role in the development of the middle ear (Martin *et al.*, 1995). All the skeletal components of this region are affected to some extent in *MHox* null mutants (Martin *et al.*, 1995). The most severely affected is the tympanic ring, completely absent in those embryos. A small deficiency is also apparent in the malleus that presents a truncated manubrium and a displaced *processus brevis*. On the contrary, the head and neck of this ossicle remain largely unaffected. The incus develops abnormally and is fused to a cartilaginous formation in the lateral skull. It is still articulated with the malleus but its long process has lost its connection with the stapes. It has been suggested that the morphology of the incus in those mutants resembles that of its reptilian homolog, namely the palatoquadrate, articulated to the malleus (or its homolog, the articular) to constitute a reptilian-like jaw joint (Martin *et al.*, 1995). Interestingly, the normal temporo-mandibular joint is absent from these mutant mice. Likewise, the stapes is also affected in a way that resembles the columella: the footplate is still inserted in the oval window but the stapedial arch has lost its foramen and remains as a column no longer articulated to the incus. On the basis of these observations it has been suggested that *MHox* is involved in the mechanisms responsible for the evolution of the vertebrate skull, and the middle ear in particular (Martin *et al.*, 1995).

The mechanism by which *MHox* directs the correct morphogenesis of the middle ear skeletal elements is not known. This gene is expressed in the mesenchyme of the branchial arches (Cserjesi *et al.*, 1992; Kuratani *et al.*, 1994) and its expression has been shown to depend on signals from the overlying epithelium (Kuratani *et al.*, 1994). Therefore, *MHox* might be a molecular mediator of the epithelial-mesenchymal interactions required for the differentiation of the precursors of the skeletal elements of the cranial region (Dunlop and Hall, 1995).

The *Dlx* gene family has also been implicated in the morphogenesis of the middle ear and in the evolutionary mechanisms that resulted in the mammalian middle ear (Qiu *et al.*, 1995,1997). In particular, the inactivation of *Dlx-2* resulted in malformations in the middle ear components that resembled non-mammalian jaw joints (Qiu *et al.*, 1995). The incus is no longer articulated to the stapes, presents an abnormal shape and remains attached to skeletal elements lateral to the basisphenoides resembling the reptilian palatoquadrate; the stapes is also columelliform. Therefore, like *MHox*, a functional *Dlx-2* is required for the proper morphogenesis of the incus and stapes (Qiu *et al.*, 1995,1997). However, unlike *MHox*, *Dlx-2* might not be involved in the formation of the malleus and tympanic ring, since neither of them are affected in the mutant animals (Qiu *et al.*, 1995,1997). Histological analysis of *Dlx-2*$^{-/-}$ embryos revealed that the stapedial artery is also absent in these mutants (Qiu *et al.*, 1997). Interestingly, the defects of *Dlx-1* mutant embryos in the ear region were restricted to the stapes and stapedial artery, which showed phenotypes similar to those of *Dlx-2* mutants (Qiu *et al.*, 1997). The rest of the area, however, remained unaltered.

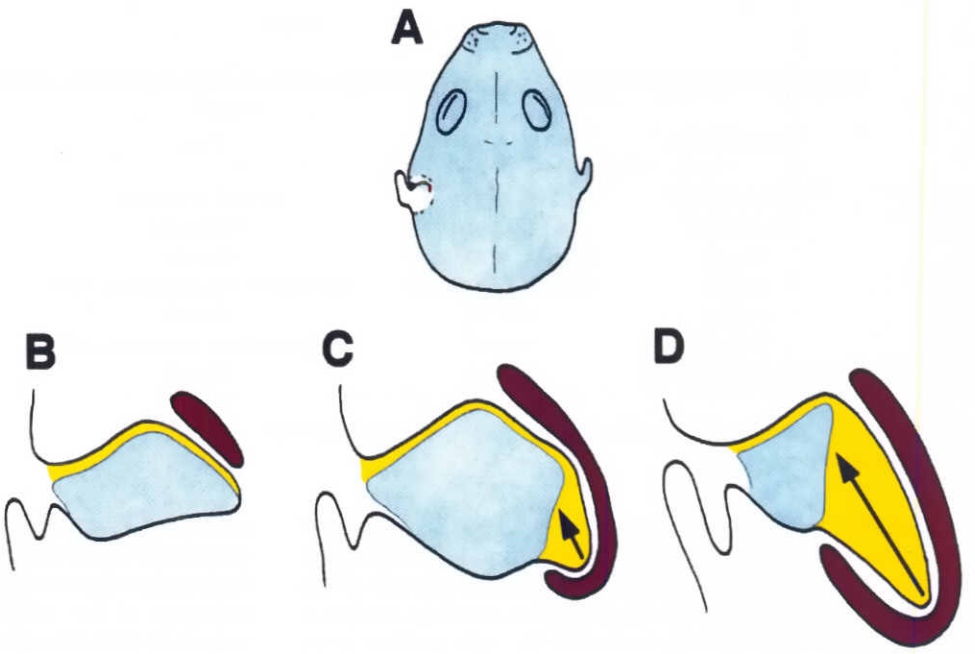

Fig. 4. Schematic representation of the formation of the lateral part of the tympanic membrane. (A) *Diagram showing the area where this process occurs.* **(B)** *The external acoustic meatus (gray) migrates toward the region of the first branchial arch, with its leading edge (yellow) directed toward the developing tympanic ring (red).* **(C)** *As the tympanic ring grows toward the region of the second branchial arch the leading edge of the external acoustic meatus follows it and starts to flatten in the plane delimited by the ring, from ventral to dorsal (direction indicated by the black arrow).* **(D)** *As development proceeds, the tympanic ring completes its formation and the external acoustic meatus fills the plane defined by the tympanic ring to form, in association with the epithelium of the middle ear cavity (not included in the scheme) the tympanic membrane.*

Another gene that has been shown to be involved in the formation of the middle ear is *gsc*. Null mutations in this gene have resulted in the complete deletion of the tympanic ring and in the truncation of the manubrium (Rivera-Pérez *et al.*, 1995; Yamada *et al.*, 1995). Also, as a consequence of the lack of tympanic ring formation, the EAM fails to develop, and, therefore, no tympanic membrane is present in these mutant animals (Rivera-Pérez *et al.*, 1995; Yamada *et al.*, 1995; Mallo and Gridley, 1996). The expression of *gsc* in the branchial area (Gaunt *et al.*, 1993) suggests that, similarly to *MHox*, it is induced in the branchial arch mesenchyme as a consequence of the epithelial-mesenchymal interactions that direct the morphogenesis of the skeleton of this area (Dunlop and Hall, 1995). In this respect it is interesting to note that, although the tympanic ring is formed from the first arch area (Mallo and Gridley, 1996), *gsc* is expressed both in the mesenchyme of the first and second branchial arches surrounding the first pharyngeal cleft (Gaunt *et al.*, 1993). The absence of *Hoxa-2* function in the second branchial arch results in the formation of an extra tympanic ring from this area without affecting the expression of *gsc* (Mallo and Gridley, 1996). This finding suggests that *Hoxa-2* might inhibit in the second branchial arch the *gsc*-mediated induction of the genes responsible for the initiation of tympanic ring morphogenesis.

The similarity of the phenotype of the *gsc* and *MHox* mutants in the skeletal components related to the tympanic membrane (i. e., tympanic ring and malleal manubrium) is remarkable (Martin *et al.*, 1995; Rivera-Pérez *et al.*, 1995; Yamada *et al.*, 1995). This similarity raises the question of whether one of the genes controls the expression of the other in this area, or whether both cooperate in the morphogenesis of those structures. The analysis of the expression of *MHox* and *gsc* in the corresponding mutant embryos, and the elucidation of the targets for those genes will be required to determine which of these possibilities is reality.

Finally, *Msx-1* might also be implicated in the formation of the middle ear, although according to *Msx-1* mutant phenotypes in mice, the role of this gene in the area might be less relevant (Satokata and Maas, 1994). *Msx-1* inactivation has resulted in quite specific effects on the development of the first branchial arch which, in the middle ear, only included the absence of the malleal *processus brevis* (Satokata and Maas, 1994).

The analysis of the importance of particular gene functions in the development of particular structures should be considered with great care. In the branchial area, for instance, many reciprocal interactions between different structures are known to be necessary for the morphogenesis of different elements (Thesleff *et al.*, 1995). Therefore, the absence of a given structure as a consequence of the inactivation of a particular gene might reflect a secondary effect rather than a direct role of the gene in the morphogenesis of this structure. A well characterized example is the failure of the EAM to invaginate in the *gsc* mutants, a defect derived from the failure of the tympanic ring to form rather than from a direct effect of the gene in the genesis of the EAM (Mallo and Gridley, 1996). It is possible that similar situations are also relevant for other mutant phenotypes discussed above. For instance, *Dlx-1* and *Dlx-2* mutant mice show malformations in the development of the stapes and in the stapedial artery (Qiu *et al.*, 1995,1997), and the development of these two structures has been shown to be embryologically coordinated (Anson and Cauldwell, 1942). It is therefore possible that *Dlx-1* and *-2* have a direct role in the morphogenesis of only one of those structures, which would then influence the formation of the other. The analysis of other embryos in which the stapes is also affected to different degrees (like *Hoxa-2*$^{-/-}$, *Mhox*$^{-/-}$ and RA treated embryos) might help to test this possibility. Also intriguing is that the manubrium of the malleus is affected in the *gsc* and *MHox* mutants without obvious effects on the rest of the ossicle, but in association with the absence of the tympanic ring (Martin *et al.*, 1995; Rivera-Pérez *et al.*, 1995; Yamada *et al.*, 1995). This might indicate a direct effect of both genes in the formation of both skeletal structures. However, it might reflect a different situation. Considering that the presence of the manubrium of the malleus (even without any contact with the rest

TABLE 1

MIDDLE EAR PHENOTYPES RESULTING FROM NULL MUTATIONS IN SEVERAL HOMEOBOX-CONTAINING GENES

	Tympanic ring	Malleus	Incus	Stapes	Tympanic membrane
Hoxa-1*	Normal or ND#	Normal or Absent	Normal or Absent	Altered orAbsent	Normal or ND#
Hoxa-2	Duplicated	Duplicated	Duplicated	Absent	Duplicated
gsc	Absent	Manubrium reduced	Normal	Normal	Absent
MHox	Absent	Manubrium reduced	Malformed and attached to skull	Arch fused	ND#
Dlx-1	Normal	Normal	Normal	Arch fused	ND#
Dlx-2	Normal	Normal	Malformed and attached to skull	Arch fused	ND#
Msx-1	Proc. brevis absent	Normal	Normal	Normal	Normal

* Two independent strains showing different phenotypes have been reported.
#ND: not determined.

of this ossicle) is always associated to a tympanic ring and EAM (Mallo, 1997), it is possible that the formation of the manubrium depends on a signal emanated from the medial surface of the external acoustic meatus. This mechanism would help to ensure that the malleal manubrium is formed in the right position to be integrated between the two epithelial layers of the tympanic membrane. In this context, the truncation of the malleal manubrium in the *gsc* and *MHox* mutants might result from the absence of a EAM consequence of the failure of the tympanic ring formation.

All the genes considered in this section contain a homeobox in their coding sequences. Therefore, as putative transcription factors, their role in the morphogenesis of the different elements of the middle ear might be to specify the spatiotemporal expression of other genes that direct the formation of bones and cartilages. They might control other master regulators of skeletal formation, molecules that specify cell-cell interactions or even structural components of the middle ear elements. The elucidation of the genes under the control of homeobox-containing transcription factors is still in a very initial phase, but some candidate targets for *Hox* genes are cell adhesion molecules like N-CAM (Jones *et al.*, 1992,1993) or l(2)gl (Tomotsune *et al.*, 1993), which could fit well in these predictions. Ironically, however, MHox was initially described as a factor able to bind and activate an enhancer from the muscle creatine kinase (Cserjesi *et al.*, 1992), which is thought to have a role in muscle but not in skeletal differentiation. It is likely that in the next few years we will witness an enormous advance in the elucidation of the physiological targets for all these homeobox genes. This knowledge will be of great help towards clarifying the molecular mechanisms for the development of the middle ear, many of which might turn out to be shared by other complex morphogenetic areas.

Summary

The middle ear is part of the sound-conducting apparatus in mammals. It contains a chain of three ossicles, the malleus, incus and stapes, located in the middle ear cavity. The ossicle chain connects the tympanic membrane, which is inserted in the tympanic ring, with the inner ear. The embryological origin of the middle ear is diverse: the skeletal elements derive from the cranial neural crest that migrate into the first two branchial arches; the middle ear cavity develops from the first pharyngeal pouch; and the tympanic membrane results from the apposition of the epithelium of the middle ear cavity with that of the external acoustic meatus, which derives from the first branchial cleft. The formation of the different structures of the middle ear results from a complex series of reciprocal interactions between the epithelium and mesenchyme of the branchial arches. The tympanic ring is induced in the mesenchyme of the first branchial arch by signals from the epithelium, and the ring induces and coordinates the formation of the tympanic membrane. Several genes have been identified that have a role in the formation of the middle ear. They include genes that are involved in skeletal formation (e.g., *Sox-9*), genes that have a general role in the development of the cranial neural crest (e.g. *Endothelin-1, AP-2, Treacle* and Retinoic acid receptors) and several homeobox-containing genes that regulate the formation of specific parts of the middle ear (e.g., *Hoxa-1, Hoxa-2, Mhox, goosecoid, Dlx-1, Dlx-2, Msx-1).*

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