Development of the mammalian ear: coordinate regulation of formation of the tympanic ring and the external acoustic meatus

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SUMMARY

The tympanic membrane in mammals is a trilaminar structure formed by the apposition of two epithelial cell layers, along with an intervening layer of cells derived from pharyngeal arch mesenchyme. One epithelial layer is contributed by the external acoustic meatus, a derivative of the first pharyngeal cleft. The other epithelial layer is contributed by the tubotympanic recess, a derivative of the first pharyngeal pouch. We demonstrate here an absolute correlation between formation of the external acoustic meatus and formation of the tympanic ring, a first arch-derived membrane bone that anchors the tympanic membrane. Experimental loss of the tympanic ring by retinoic acid treatment, or duplication of the ring in Hoxa-2 null mutant embryos, resulted in corresponding alterations in formation of the external acoustic meatus. We suggest that the tympanic ring primordium induces formation and morphogenesis of the external acoustic meatus, and that expression of the Hoxa-2 and goosecoid genes may be involved in regulating the formation and morphogenesis of these structures.

Key words: tympanic ring, Hoxa-2, goosecoid, external acoustic meatus, retinoic acid, mouse, ear

INTRODUCTION

The formation of the mammalian ear has been the object of intense study since the early nineteenth century, and some of the most striking characteristics distinguishing the mammalian skull from that of other vertebrates are seen in the structure of the middle ear (de Beer, 1937; Wake, 1979; Hall, 1992; Kent, 1992; Novacek, 1993). Operationally, the mammalian ear can be subdivided into three interconnected, but relatively discrete, units: the external, middle and inner ear (Fig. 1). The external ear consists of the pinna, or external flap of the ear; the external acoustic meatus, or ear canal; and the tympanic membrane or eardrum. The tympanic membrane abuts the cavity of the middle ear, which is traversed by a chain of three auditory ossicles (the malleus, incus and stapes). These small bones serve to conduct vibrations from the tympanic membrane across the middle ear cavity and transmit them into the inner ear, which contains the sensory organs for both hearing and balance.

Embryonically, the external and middle ears are derived from the first and second pharyngeal arches, the first pharyngeal cleft and the first pharyngeal pouch, while the inner ear is derived from the otic vesicle (Moore and Persaud, 1993). The external acoustic meatus forms from the first pharyngeal cleft by the ingrowth of a solid epithelial plate of ectodermal cells called the meatal plug. Late in development, cells in the center of the plug degenerate, leaving a cavity lined by ectodermally derived epithelial cells. The middle ear cavity forms from the expansion and elongation of the endodermal lining of the first pharyngeal pouch to form the tubotympanic recess. The tympanic membrane is a trilaminar structure that forms from the juxtaposition of the epithelial layers of the external acoustic meatus and the tympanic ring, a first arch-derived membrane bone that anchors the tympanic membrane. Experimental loss of the tympanic ring by retinoic acid treatment, or duplication of the ring in Hoxa-2 null mutant embryos, resulted in corresponding alterations in formation of the external acoustic meatus. We suggest that the tympanic ring primordium induces formation and morphogenesis of the external acoustic meatus, and that expression of the Hoxa-2 and goosecoid genes may be involved in regulating the formation and morphogenesis of these structures.
of body weight. Genotyping of the embryos resulting from intercrosses was performed by PCR as described (Gendron-Maguire et al., 1993; Rijli et al., 1993). We observed an absolute correlation between the presence of the tympanic ring and the formation of the external acoustic meatus, suggesting that formation of the tympanic ring regulates formation and growth of the external acoustic meatus.

RESULTS

The analysis of gsc null mice indicated that expression of gsc has a role in the morphogenesis of the tympanic membrane. In these mice (Rivera-Pérez et al., 1995; Yamada et al., 1995), the tympanic membrane did not form properly because the external acoustic meatus failed to develop. Also, the bone that supports the tympanic membrane, the tympanic ring, was not present in these mutants. We were interested in determining the role of gsc in morphogenesis of the tympanic membrane. The expression of gsc in the region of the first pharyngeal cleft, which is where the external acoustic meatus originates, is restricted to the mesenchyme and is absent from the overlying epithelium (Gaunt et al., 1993; also see Fig. 5E). Since gsc is not expressed in the cells directly contributing to formation of the external acoustic meatus, we hypothesized that, in the gsc mutants, the absence of the external acoustic meatus might be secondary to the failure to develop a tympanic ring. From this hypothesis, we would predict that the tympanic ring and the external acoustic meatus must be formed in a coordinate way. We therefore tested whether experimental manipulation of formation of the tympanic ring would result in corresponding alterations in formation of the external acoustic meatus.

Formation of the tympanic ring and external acoustic meatus

We began these studies by analyzing the relationships among the different structures involved during morphogenesis of the middle ear (Figs 2, 3). A primordium for the tympanic ring first becomes apparent by 13.5 dpc as a condensation ventral to the first pharyngeal cleft, lateral to Meckel’s cartilage (Fig. 2A-C). As development progresses, this condensation extends first dorso-caudally, then rostrally, resulting in a C-shaped structure. Ossification is first detected in the part of the condensation adjacent to Meckel’s cartilage (Fig. 3A-D, and data not shown), then progresses through the rest of the condensation to form a fully developed tympanic ring that is evident by 17.5 dpc (Fig. 4A, and data not shown).

Concomitantly, the external acoustic meatus initially forms as a collapsed sac-shaped epithelial invagination, the meatal plug, that originates in the region of the first pharyngeal cleft and progresses ventrally and caudally towards the developing tympanic ring (Figs 2B,C, 3B-D). Later in development, while the edge of the meatal plug is still pointing to the tympanic ring, its medial surface moves towards the tubotympanic recess and finally attaches to its lateral epithelium to constitute the tympanic membrane (Figs 3C,D, 4A). This analysis of wild-type embryos demonstrates the intimate relationship between the development of the tympanic ring and the external acoustic
meatus, and is compatible with the hypothesis that formation of the tympanic ring and formation of the external acoustic meatus are coordinately regulated.

**Formation of the external acoustic meatus is dependent on formation of the tympanic ring**

We then analyzed formation of the external acoustic meatus in embryos in which formation of the tympanic ring had been experimentally altered. For these experiments, we took advantage of the finding that homozygous Hoxa-2 mutant embryos exhibit a mirror-image duplication of the tympanic ring with complete penetrance (Gendron-Maguire et al., 1993; Rijli et al., 1993). If formation of the tympanic ring and formation of the external acoustic meatus are in fact coordinately regulated, we would predict that the external acoustic meatus would be duplicated in Hoxa-2 mutant embryos. Analysis of Hoxa-2 mutants showed that this was indeed the case and an abnormal tympanic membrane resulted from this duplication (Fig. 4B).

At 13.5 and 15.5 dpc, two meatal plugs (the embryonic precursor of the external acoustic meatus) originated from the first pharyngeal cleft in the mutant embryos (Figs 2D, 3E). Each of these progressed towards two independent tympanic ring primordia, located respectively ventral and dorsal to the cleft (Figs 2E,F, 3E-H). This resulted in two meatal plugs, each containing a medial and a lateral surface. The subsequent aposition of the medial surfaces of these structures and the lateral epithelium of the tubotympanic recess resulted in the formation of a dual tympanic membrane that left the manubrium of the malleus between the two external acoustic meatus instead of in its normal position, between the external acoustic meatus and the tubotympanic recess (compare Fig. 4A and B). These results further indicate the involvement of the tympanic ring in the morphogenesis of the external acoustic meatus.

We then took advantage of the finding that maternal administration of retinoic acid results in loss of the tympanic ring in a large proportion of the resulting embryos (Kessel, 1992). We therefore treated pregnant females with retinoic acid at day 8 plus 5 hours of gestation. Consistent with the previous report (Kessel, 1992), this treatment resulted in loss of the tympanic ring in treated embryos (Fig. 4D, and data not shown), although the penetrance of this effect was not complete. Histological analysis of these embryos at 17.5 dpc showed that, associated with this skeletal deficiency, there was a complete absence of the external acoustic meatus (Fig. 4D, and data not shown). We have never detected an external acoustic meatus in mice lacking tympanic rings. The incomplete penetrance of the retinoic acid treatment in fact provided an excellent internal control for these findings. Several retinoic acid-treated embryos had unilateral, rather than bilateral, loss of the tympanic ring (Fig. 4C,D). When these embryos were sectioned, an external acoustic meatus was detected on the side where the tympanic ring was present (Fig. 4C) but not where it was absent (Fig. 4D). These results provide strong support for the hypothesis that formation of the tympanic ring and formation of the external acoustic meatus are coordinately regulated.

**gsc expression in retinoic acid-treated and Hoxa-2 mutant embryos**

The data presented so far support the hypothesis that the absence of the external acoustic meatus in gsc null mutants (Rivera-Pérez, 1995; Yamada et al., 1995) is secondary to the absence of the tympanic ring. Since formation of the tympanic ring is affected in both Hoxa-2 and gsc mutant mice, we asked whether duplication or loss of the tympanic ring in the experimentally manipulated embryos might be mediated through an alteration of gsc expression. To address this issue, we analyzed

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**Fig. 2.** Development of the external acoustic meatus of wild-type and Hoxa-2−/− mice at 13.5 dpc. Transverse sections of the otic region in wild-type (A-C, progressing rostral to caudal) and Hoxa-2−/− mice (D-F, from rostral to caudal) were stained with hematoxylin and eosin. In the wild-type embryos, only one meatal plug (the embryonic precursor to the external acoustic meatus; long arrow) forms, directed towards the mesenchymal condensation that will give rise to the tympanic ring (short arrow). In these embryos, only one Meckel’s cartilage (M) and one Reichert’s cartilage (R) are present on each side of the head. In the Hoxa-2−/− embryos, two meatal plugs form (long arrow for the one in the normal position, and large arrowhead for the duplicated one), each directed towards the corresponding normal (short arrow) and duplicated (small arrowhead) mesenchymal condensations from which the two tympanic rings will develop. A duplicated Meckel’s cartilage (M and M′) can be seen in these mutants along with the absence of the Reichert’s cartilage. In all panels, embryo sections are oriented with the ventral side (snout) towards the top of the figure.
gsc RNA expression in wild-type, Hoxa-2 mutant and retinoic acid-treated embryos by whole-mount in situ hybridization (Fig. 5). In the craniofacial region of wild-type embryos at 11.5 dpc, gsc is expressed in three domains that correlate very well with the regions affected in the gsc null-mutant mice: in mesenchyme surrounding the nasal pits, in the mandibular arch and at the base of the first pharyngeal cleft (Fig. 5A). This expression was not affected in the Hoxa-2 mutant embryos, indicating that the abnormal morphogenesis of the tympanic ring observed in these mutants is not exerted through an effect on gsc transcription (Fig. 5B). In retinoic acid-treated embryos, there was a variable reduction in the intensity and extent of gsc RNA expression, particularly in the region surrounding the first pharyngeal cleft (Fig. 5C,D). While retinoic acid can have multiple effects on treated embryos (e.g., Lee et al., 1995), these results are consistent with the notion that gsc expression plays a role in formation of the tympanic ring, and the variable reduction in gsc RNA expression observed in the treated embryos may be the cause of the incomplete penetrance of retinoic acid-mediated inhibition of tympanic ring formation.

DISCUSSION

A morphogenetic connection between formation of the tympanic ring and formation of the external acoustic meatus was suggested from our analysis of gsc null mutant mice, which lack both structures (Rivera-Pérez et al., 1995; Yamada et al., 1995). The studies described here strongly suggest that morphogenesis of the external acoustic meatus and the tympanic ring are spatially and temporally integrated. This conclusion is supported by the fact that two additional and complementary alterations in formation of the tympanic ring resulted in corresponding alterations in formation of the external acoustic meatus. The first alteration was induced by exposing embryos to retinoic acid under conditions that prevent development of the tympanic ring (Kessel, 1992). In addition to lacking tympanic rings, the treated embryos also did not form an external acoustic meatus. These same experimental conditions provided an excellent internal control, since the effect of the retinoic treatment on tympanic ring formation was not completely penetrant. In treated embryos exhibiting only unilateral loss of the tympanic ring, an external acoustic meatus formed only on the side of the head where the tympanic ring was present, and never on the side where the tympanic ring was absent (Fig. 4C,D).

A complementary experimental alteration in tympanic ring formation resulted from a null mutation in the Hoxa-2 gene. Mice homozygous for this mutation contain a mirror-image duplication of the tympanic ring as a consequence of the
reprogramming of second pharyngeal arch skeletogenic neural crest towards a first arch identity (Gendron-Maguire et al., 1993; Rijli et al., 1993). The Hoxa-2 mutant embryos formed two external acoustic meatus on each side of the head, which resulted in formation of an abnormal tympanic membrane.

Mutations in other genes also affect formation of the tympanic ring. For example, mice homozygous for a null mutation in the Mhox gene (which encodes a homeobox-containing protein) lack tympanic rings, as well as numerous other cranial skeletal elements (Martin et al., 1995). This deficiency in the Mhox mutant mice is probably the result of a general defect in the growth of chondrogenic and osteogenic precursors that most severely affects derivatives of the first pharyngeal arch (Martin et al., 1995). Severe craniofacial defects, including absence of the tympanic ring, are also observed in mice homozygous for a mutation in the Endothelin-1 gene (which encodes a peptide vasoconstrictor secreted by endothelial cells, as well as many other cell types) (Kurihara et al., 1994). Consistent with our findings in the gsc mutant and retinoic acid-treated embryos, the external acoustic meatus is also absent in the Endothelin-1-deficient mice (Kurihara et al., 1994); in the Mhox mutants, presence or absence of the external acoustic meatus was not reported (Martin et al., 1995).

Two models could explain this correlation between presence of the tympanic ring and formation of the external acoustic meatus. In the first model, this correlation results from the positional specification of the neural crest cells that populate the first and second pharyngeal arches. In this case, development of the tympanic ring and external acoustic meatus are coordinately regulated due to spatial programming of the cranial neural crest, which most likely occurs prior to colonization of the pharyngeal arches (Gendron-Maguire et al., 1993; Rijli et al., 1993). This model would suggest that cranial neural crest is spatially reprogrammed in retinoic acid-treated embryos and in Hoxa-2, gsc and Endothelin-1 mutant mice.

In the second model, coordinate regulation of formation of the tympanic ring and external acoustic meatus is due to direct induction of formation of the external acoustic meatus by the tympanic ring (or by the mesenchymal condensation that gives rise to the tympanic ring). While our data are compatible with either model, we favor the interpretation that the tympanic ring directly induces formation and morphogenesis of the external acoustic meatus. While respecification of the positional identity of the cranial neural crest has clearly occurred in Hoxa-2 mutant mice, many of the skeletal defects observed in retinoic acid-treated, gsc mutant and Endothelin-1 mutant mice seem more likely to be due to retarded formation and growth of chondrogenic and osteogenic precursors. In addition, while all of the experimental regimes that result in alterations in tympanic ring formation (Hoxa-2 mutation, gsc mutation, Endothelin-1 mutation, and maternal retinoic acid administration) also affect other cranial structures, none of the embryos produced by one regime are identical to embryos produced by another regime (i.e., Hoxa-2 mutant embryos, gsc mutant embryos and retinoic acid-treated embryos are readily distinguishable from one another). However, in all these experimental regimes formation of the external acoustic meatus is correlated with presence of the tympanic ring, even on either side of the head of an individual embryo (i.e., retinoic acid-treated embryos with unilateral loss of the tympanic ring; Fig. 4C,D). This suggests to us that the correlation between formation of the tympanic ring and the external acoustic meatus is a direct effect due to induction of the external acoustic meatus by the tympanic ring.

The diminution of gsc RNA expression in retinoic acid-treated embryos, along with the alterations in formation and morphogenesis of the tympanic ring and external acoustic meatus in Hoxa-2 and gsc mutants, suggests that expression of these genes may be involved in regulating formation of these

![Fig. 4. Effect of the Hoxa-2 mutation and retinoic acid treatment on the otic region at 17.5 dpc. Frontal sections through the otic region of wild-type (A), Hoxa-2−/− (B) and retinoic acid-treated (C,D) 17.5 dpc embryos were stained by the alcan blue/chlorantine fast red method, which differentiates membranous (red) versus endochondral (blue-green) bone. C and D show two sides of the same retinoic acid-treated embryo, which had undergone unilateral loss of the tympanic ring. (A) In the wild type, a single meatal plug (e) had formed, directed towards the tympanic ring (T). The meatal plug had attached to the tubotympanic recess (t), leaving the manubrium of the malleus (m) in between. (B) In the Hoxa-2−/− embryo an additional meatal plug had formed (e′), directed towards the duplicated tympanic ring (T′). The manubrium of the malleus (m) is left between the two meatal plugs (e,e′). (C and D) In this retinoic acid-treated embryo, a meatal plug (e) forms only on the side of the embryo where the tympanic ring is present (C), but not on the side where it is absent (D). M, Meckel’s cartilage.](image-url)
structures. In addition, since the skeletal alterations in the otic region of Mhox, Endothelin-1 and Hoxa-2 mutant mice are more severe than the skeletal alterations in the otic region of gsc mutant mice, gsc expression may play a more direct role than expression of these other genes in regulating formation and morphogenesis of these structures.

In the pharyngeal arches, the gsc gene is expressed in portions of the first and second arches (Gaunt et al., 1993; also see Fig. 5), while the Hoxa-2 gene is expressed in the second and third arches, but is not expressed in the first arch (Dolle et al., 1993; Krumlauf, 1993; Prince and Lumsden, 1994). We suggest that gsc-expressing mesenchymal cells in the first pharyngeal arch contribute to the formation of the mesenchymal condensation that eventually forms the tympanic ring. Thus, in this model the primary effect of the gsc mutation on morphogenesis of the otic region is on formation of the tympanic ring; absence of the external acoustic meatus is a secondary effect. We also propose that expression of Hoxa-2 inhibits the potential of gsc-expressing mesenchymal cells to form a tympanic ring. Therefore, the primordium of the tympanic ring forms in the first arch portion of the gsc-expression domain, which does not express Hoxa-2. However, expression of Hoxa-2 prevents gsc-expressing mesenchymal cells located in the second pharyngeal arch from forming a tympanic ring. This model could also explain why gsc expression is unaltered in Hoxa-2 mutant embryos (Fig. 5B), despite the duplication of the tympanic ring that takes place in these mutants.

In summary, our data indicate that morphogenesis of the external acoustic meatus and the tympanic ring are coordinate regulated. At present we have no data regarding the mechanistic aspects of this process. Further experiments will be required to determine the nature of the signal or signals involved in coordinating the morphogenesis of the otic region in mice.

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REFERENCES


Formation of external acoustic meatus


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