Development 127, 4127-4136 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV4429

Assembling a functional tympanic membrane: signals from the external acoustic meatus coordinate development of the malleal manubrium

Moisés Mallo[1,*], Heinrich Schrewe[1], James F. Martin[2], Eric N. Olson[3] and Sabine Ohnemus[1]

[1]Department of Developmental Biology, Max-Planck Institute of Immunobiology, Stübeweg 51, D-79108 Freiburg, Germany
[2]Alkek Institute of Biosciences and Technology, Texas A&M System Health Science Center, 2121 Holcombe Boulevard, Houston, TX 77030, USA
[3]Dept. of Molecular Biology and Oncology, UT Southwestern Medical Center in Dallas, 5323 Harry Hines Boulevard, Dallas, TX 75235-9148, USA
*Author for correspondence (e-mail: mallo@immunbio.mpg.de)

Accepted 20 July; published on WWW 7 September 2000

SUMMARY

In terrestrial mammals, hearing starts with the perception of acoustic pressure by the tympanic membrane. Vibrations in this membrane are then transduced into the inner ear by the ossicle chain of the middle ear, composed of the malleus, incus and stapes. The proper connection of the ossicle chain with the tympanic membrane, provided by the insertion of the manubrium of the malleus into the eardrum, is essential for the functionality of the hearing apparatus. We describe here the mechanisms regulating the development of the manubrium and its integration into the tympanic membrane. We show that the external acoustic meatus (EAM), which eventually forms the outer epithelium of the tympanic membrane, plays an essential role in this developmental process. Histological and expression analyses indicate that the manubrium develops close to the EAM with a similar temporal sequence. In addition, when the middle ear ossicles are allowed to develop in vitro under conditions that do not support further EAM development, the manubrium develops only up to the stage of its induction at the time of explantation. Moreover, genetically or teratogenically derived alterations in the EAM also have an effect on manubrial development. Finally, we show that the EAM is the source of two quite opposite activities, one that induces chondrogenesis and another that represses it. The combination of these two activities results in the proper positioning of the manubrium.

Key words: Middle ear, Tympanic membrane, *Sox9*, *Prx1*, Mouse

INTRODUCTION

Morphogenesis of complex structures involves a series of coordinated mechanisms to ensure both correct patterning and formation of the individual elements as well as integration of all those processes to assemble functional structures. The study of the latter is often hindered by the complexity of the structures themselves. Therefore, analysis of relatively simple structures can help to uncover the basic mechanisms coordinating development of the different individual components. One such system is the apparatus that transmits vibrations from airborne sound into the inner ear in mammals. This apparatus, composed essentially of the tympanic membrane, also called the eardrum, and the middle ear, assembles elements derived from all three germ layers but is still simple enough to allow experimental analysis of its developmental mechanisms (Carlson, 1994; Mallo, 1998).

Vibrations in the tympanic membrane must be transduced and amplified into the endolymphatic fluid of the cochlea, where mechanoreceptors convert those vibrations into nervous impulses. The connection between the tympanic membrane and the inner ear is provided by a chain of three ossicles, the malleus, the incus and the stapes, which form a bridge between the two structures (Fig. 1). The connections of the ossicle chain to the eardrum and inner ear are made by the manubrium of the malleus, inserted in the tympanic membrane, and the stapedial footplate located in the oval window (Carlson, 1994; Mallo, 1998). In addition to these basic elements, other structures are essential for the functionality of the system, in particular the tympanic ring, which provides support to the tympanic membrane, and muscles that modulate the intensity of the vibrations transmitted by the ossicles.

The embryological origin and histological characteristics are different among the various elements of this structure (Carlson, 1994; Mallo, 1998). The tympanic membrane results from the apposition of two epithelia, provided by the ectoderm of the first branchial cleft and the endoderm of the first pharyngeal pouch, leaving inbetween a fibrous layer of mesenchymal origin (Fig. 1B). The three middle ear ossicles develop by endochondral ossification from neural crest-derived mesenchyme in the proximal part of the first (malleus and incus) or second (stapes) branchial arches (Carlson, 1994; Mallo, 1998). The tympanic ring is formed in the first branchial arch by dermal ossification of the neural crest-derived

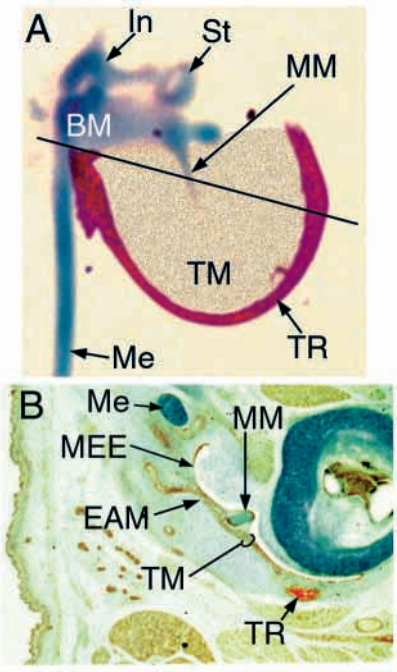

Fig. 1. Mouse middle ear at E18.5. (A) Skeletal staining. The tympanic membrane (TM), represented as a brown shadow, is supported by the tympanic ring (TR). The manubrium of the malleus (MM) connects the ossicle chain with the tympanic membrane. The incus (In) is articulated with the body of the malleus (BM) and with the stapes (St). These three elements, malleus, incus and stapes, form the middle ear ossicle chain. At this stage, the body of the malleus is still attached to Meckel's cartilage (Me). (B) Histological section at the level indicated in A. The tympanic membrane is formed by the apposition of the external acoustic meatus (EAM) and the middle ear epithelium (MEE). The manubrium of the malleus is located between the two epithelia.

mesenchyme (Novacek, 1993). The otic capsule, which provides the skeletal support for the inner ear, is mainly of mesodermal origin, although at least part of the walls of the oval window may possibly originate from neural crest cells (Couly et al., 1993). Finally, the muscles that insert in the ossicles derive from cranial paraxial mesoderm (Noden, 1988).

Recent molecular and genetic experiments have provided an enormous advance in our understanding of the genes and mechanisms controlling patterning and formation of some of the individual elements of the middle ear (reviewed in Mallo, 1998). Less is known about how the different elements of the middle ear are assembled together. Proper location of the tympanic membrane in the auditory canal seems to be directed by the tympanic ring (Michaels and Soucek, 1989; Mallo and Gridley, 1996). In addition, Köntges and Lumsden (1996) have shown that, at least for the chicken, proper insertion of the facial muscles might be directed by some neural crest cells that locate at the insertion site in the skeletal structures. Therefore, it is possible that a similar mechanism applies for the tensor tympani and stapedial muscle.

Previous analyses of *Gsc* null mutants showed that the manubrium of the malleus is very hypomorphic in these embryos (Rivera-Pérez et al., 1995; Yamada et al., 1995), suggesting a role for *Gsc* in the development of this structure. Gene expression data and chimeric studies were in agreement with this possibility because *Gsc* is expressed in the manubrium and $Gsc^{-/-}$ cells seem to be excluded from this structure in the chimeras (Rivera-Pérez et al., 1999). However, the same was true for the rest of the malleus, which is not affected by the *Gsc* null mutation, suggesting that other mechanisms might be involved in the genesis of this phenotype. In this paper we have analyzed the development of the manubrium using a combination of in vivo and in vitro experimental approaches. Our results indicate that the EAM plays an essential role in the induction and proper location of this structure by providing a combination of chondrogenic-inducing and repressing activities that coordinate development of the manubrium in the underlying mesenchyme.

MATERIALS AND METHODS

Mouse strains and analyses of embryos

The *Prx1* and the *Acvr2* mutant mice have been previously described (Martin et al., 1995; Song et al., 1999). For the retinoic acid treatments, mice were mated for 2 hour periods. When plugs were detected the half-time of the mating period was considered as the time of fertilization. All-*trans*-retinoic acid was administered at day 8 plus 6 hours, as described (Mallo, 1997).

Skeletal preparations were performed as described previously (Mallo and Brändlin, 1997). For histological analysis, embryos were fixed in Carnoy's, dehydrated, embedded in paraffin, sectioned at 10 μm and stained with Hematoxylin and Eosin, or with Alcian Blue-Chlorantine Fast Red, as described (Mallo and Gridley, 1996).

Non-radioactive in situ hybridization was performed on sections from paraformaldehyde-fixed and paraffin-embedded tissue as described in Kanzler et al. (1998). The probes for *Fgf4* (Niswander and Martin, 1992), *Fgf9* (Colvin et al., 1999), *Bmp4* (Jones et al., 1991), *Sox9* and *Prx1* (Kanzler et al., 1998) have previously been described.

Middle ear cultures

The age of the donor embryos used for these experiments was estimated according to morphological parameters of the pinna and EAM, essentially as described in Miyake et al. (1996a). The region of the embryo containing the mesenchyme from which the middle ear bones originate was dissected out in incubation medium (bicarbonate-free DMEM containing 25 mM Hepes, pH 7.2, 15% fetal calf serum, 50 units/ml penicillin and 50 μg/ml streptomycin). The caudal limit of this piece was the pinna, which was not included in the explants; the rostral limit was located approximately 1 mm rostral to the EAM, and the other two limits of the explant were cut perpendicular to the pinna from the end of this structure. In addition, the developing otic capsule was not included in the explants. After incubation at room temperature for 15 minutes, the tissues were placed on polycarbonate membranes (1.0 μm pore size) on top of metal grids in contact with incubation medium and kept at 37°C in an atmosphere containing 5% CO_2 and 95% humidity. Explants from day 12 (E12) embryos were incubated for 3 days and those from E13 for 2 days. After incubation, explants were removed from the membrane and fixed in 100% ethanol overnight. Staining of the skeletal elements was performed essentially as for the embryos, but with reduced clearing times.

Recombination experiments

First branchial arches from E10.5 embryos were dissected out in PBS and incubated with 2% trypsin/pancreatin in Tyrode's salt solution, on ice for 26 minutes. The enzymes were then blocked with incubation medium (as above). Epithelium and mesenchyme were then separated manually. The medial epithelium of the external acoustic meatus was obtained from the ear region of E13.5 embryos (see above), which

were treated with trypsin and pancreatin similarly to the first branchial arches. The first arch mesenchymes were laid on top of polycarbonate membranes and the epithelia placed in contact with the appropriate area of the mesenchyme. The filters were then incubated on top of metal grids in contact with incubation medium for 2 days at 37°C, in an atmosphere containing 5% CO_2, 95% humidity. After incubation, the filters were then soaked briefly in methanol and the tissues fixed overnight at 4°C in 4% paraformaldehyde. The explants were then analyzed by whole-mount in situ hybridization as described in Kanzler et al. (1998).

RESULTS

Development of the middle ear ossicles in vitro

To begin investigating the mechanisms of malleal manubrium development we took advantage of the ability of the middle ear elements to undergo at least partial development in vitro in a culture system. In a series of preliminary experiments we found culture conditions that allowed the prospective middle ear region from embryos at stages when middle ear ossicles are still undetectable to complete development of endochondral middle ear elements (not shown). Analysis of the ossicles obtained in explants from E12.25 to E13.25 embryos revealed that, while the rest of the malleus looked fairly normal, the size of the manubrium was clearly different among the various cultures, according to the age of the donor embryo (Fig. 2). While in explants from E12.25 embryos this element was very small (Fig. 2A), in those from E13.25 it was fully grown (Fig. 2C); explants from embryos of intermediate ages showed intermediate levels of manubrial development (Fig. 2B). These results suggested that the mesenchyme from E12.25 embryos did not contain all the program required to develop the manubrium, and that this program was sequentially provided between this time and E13.25. Histological analysis of the ear region at those stages indicated that this is the initial period of development of the external acoustic meatus (EAM) (Fig. 3; and not shown). Analysis of sections of E12.25 ear explants at different incubation times indicated that the EAM did not undergo substantial development under these culture conditions (Fig. 2D,E), maybe due to physical constraints. Therefore, it is possible that the EAM could play a role in the sequential induction of the manubrium between E12.25 and E13.25.

Temporal correlation of EAM invagination and manubrium induction

To determine if the EAM could be involved in manubrial morphogenesis, we first studied how the spatial and temporal induction of this element correlated with EAM morphogenesis. In these experiments, we estimated cartilage induction by *Sox9* expression, as an early marker of cartilage differentiation (Wright et al., 1995). The manubrium is located at the caudal end of the malleus, parallel to the main axis (proximodistal) of Meckel's cartilage (Fig. 1A). At E12.25 the EAM is starting to develop as an invagination of the first branchial cleft into the first arch region (Fig. 3A,C,E). At this stage the caudal end of the developing malleus is located caudal to the early primordium of the EAM (Fig. 3A,C,E). The proximodistal extension of the *Sox9* domain in this area is very small and does not reach farther than the EAM (Fig. 3E). At E13.25, however, when the tip of the EAM has almost completed invagination along the whole semicircumference, the *Sox9* signal corresponding to the developing manubrium extends distally from the caudal extremity of the malleus, and is associated with the medial epithelium of the EAM (Fig. 3B,D,F). Thus, cartilage induction in the region corresponding to the manubrium correlates both spatially and temporally with the development of the EAM. Therefore, it seems possible that mechanisms exist that coordinate development of both structures.

Coordinated development of the EAM and the malleal manubrium in vivo

The above results suggest that the EAM might play a role in manubrial development. If this is indeed the case, alterations in their development might also be connected. To test this, we analyzed several situations of middle ear dismorphogenesis resulting from either particular gene mutations or drug-induced teratogenesis.

The middle ear phenotype of *Gsc*$^{-/-}$ mutants includes absence of both the EAM and the manubrium (Rivera-Pérez et al., 1995; Yamada et al., 1995), consistent with the first structure playing a role in the development of the second. Inactivation of the *Prx1* gene (previously known as *MHox*) also shows malformations in the malleus that are restricted to the manubrium (Martin et al., 1995). Histological analysis of the middle ears of *Prx1*$^{-/-}$ embryos revealed that the EAM is also missing in these mutants (Fig. 4), in keeping with a possible role of the EAM in manubrial development. Moreover, the manubrium is essentially the only endochondral element clearly hypomorphic in *Prx1*$^{-/-}$ embryos (Martin et al., 1995) and expression of *Prx1* appears to be downregulated in the condensing manubrium (see below and Fig. 9B), similar to

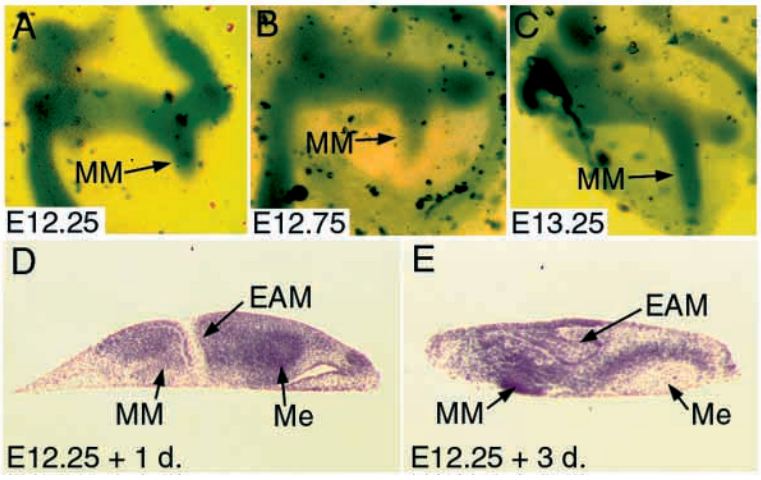

Fig. 2. In vitro development of the middle ear ossicles. (A-C) The prospective middle ear region was dissected out at E12.25 (A), E12.75 (B) and E13.25 (C), cultured in vitro and the ossicles stained with Alcian Blue. The size of the manubrium of the malleus (MM) is bigger in explants from later stages than in those from younger embryos. (D,E) Histological sections through E12.25 explants after 1 day (D) or 3 days (E) of incubation. Under these culture conditions, the external acoustic meatus (EAM) does not develop as in the embryo. Me, Meckel's cartilage.

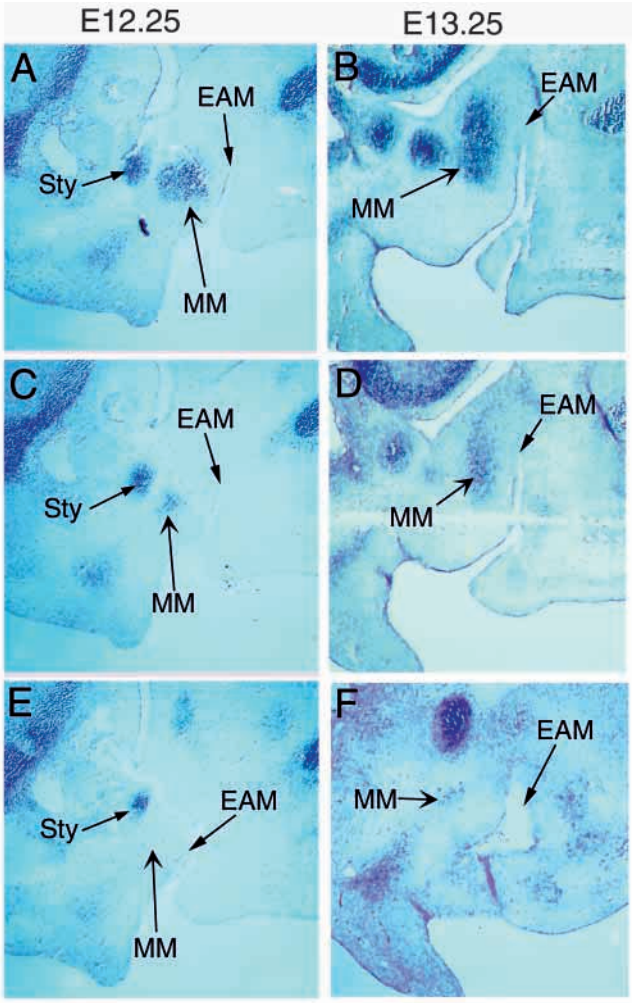

Fig. 3. Induction of the malleal manubrium during mouse embryo development. Transverse sections through the middle ears of E12.25 (A, C, E) and E13.25 (B, D, F) mouse embryos were hybridized with an antisense probe for *Sox9*. At both stages sections corresponding to different proximodistal levels are shown, with A and B being the most proximal and E and F the most distal sections. (A,C,E) At E12.25 the invagination of the external acoustic meatus (EAM) is still very small. Induction of the malleal manubrium (MM), as estimated by *Sox9* induction, is detected medial to the EAM, and distally it does not extend further than the distal end of the EAM. (B,D,F) At E13.25 the invagination of the EAM is almost complete. The induction of the manubrium is also extended distally, medial to the EAM, but it does not extend further than the EAM. The sections are oriented with the rostral side to the right. Sty, styloid process.

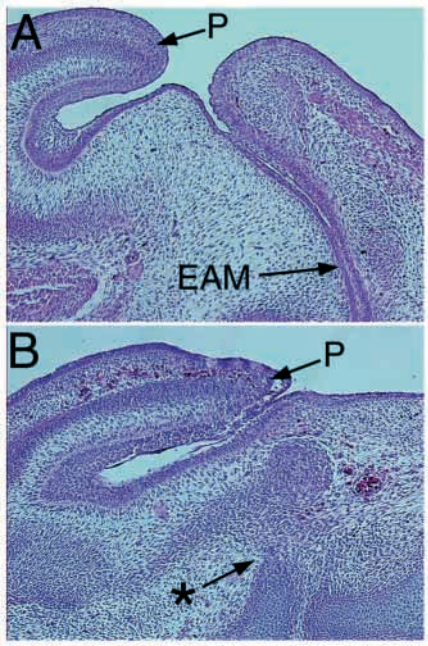

Fig. 4. Absence of external acoustic meatus in *Prx1* mutant embryos. Transverse sections through the external ear of wild-type (A) and *Prx1*$^{-/-}$ (B) E16.5 embryos. The external acoustic meatus (EAM) is present in wild-type embryos but is absent from the *Prx1* mutants (asterisk). The sections are oriented with the rostral side to the right. P, pinna of the external ear.

what has been described for other endochondral elements, including the rest of the malleus (Cserjesi et al., 1992; Kuratani et al., 1994; our unpublished results).

A rather complementary picture was provided by retinoic acid (RA) teratogenesis. Under specific conditions of treatment, this drug produces a variety of middle ear abnormalities in developing embryos (Mallo, 1997; Zhu et al., 1997). The affected structures include the EAM, which is only detected in middle ears containing a tympanic ring (Mallo and Gridley, 1996). To see if the development of the manubrium also correlated with that of these two structures, we treated pregnant females with RA and analyzed the middle ear skeleton of affected embryos. When the tympanic ring was present, a cartilage was also found located in the plane determined by the ring, with a shape reminiscent of a malleal manubrium and with a processus brevis attached to it (Fig. 5A). This cartilage was present even in cases when the rest of the malleus was severely affected (Fig. 5A). Conversely, we never found such a structure in middle ears that did not contain tympanic rings (not shown). Histological analyses of RA-treated embryos indicated that these manubrium-looking cartilages were associated with the medial surface of the EAM (Fig. 5B; Mallo 1997), similar to manubriums in wild-type embryos. These results are consistent with a direct morphogenetic correlation between the manubrium and the EAM.

Mutant mice for the activin receptor type IIA gene (*Acvr2*) show variable deficits in first branchial arch development with incomplete penetrance (Matzuk et al., 1995; and not shown). In extreme cases the mandibular skeleton, represented by the Meckel's cartilage and the dentary bone, is totally absent (not shown). However, in those embryos tympanic rings were present, although medially displaced and fused with each other in the midline (Fig. 6A). The malleus was still identifiable on both sides, linked to the contralateral through a thin cartilaginous bridge (Fig. 6A). Interestingly, the manubrium of the malleus was fully developed and correctly located with respect to the tympanic ring. Histological analysis of the middle ear region revealed that EAMs were also present, associated with both the tympanic ring and the malleal manubrium (Fig. 6B). The EAMs were very long, most likely as a result of the medial displacement of the tympanic ring.

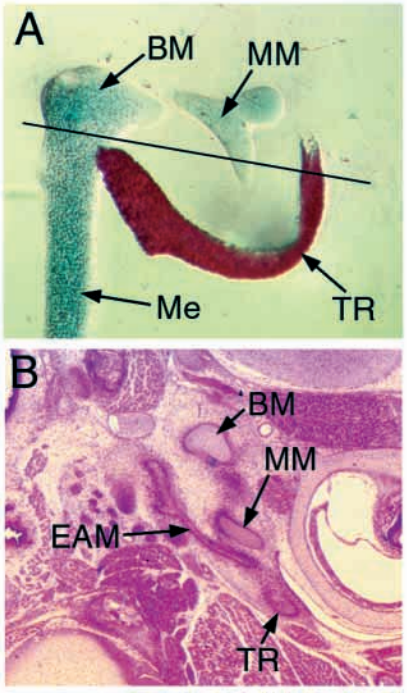

Fig. 5. Middle ear of E18.5 embryos that had been treated with retinoic acid at day 8 plus 6 hours. (A) Skeletal staining. The malleus is strongly hypomophic, with a small body (BM) and no neck. A cartilage resembling the malleal manubrium (MM) is detected in the plane defined by the tympanic ring (TR). (B) Histological sections at the level indicated in A reveal the presence of an external acoustic meatus (EAM); the manubrium of the malleus is associated with its medial surface.

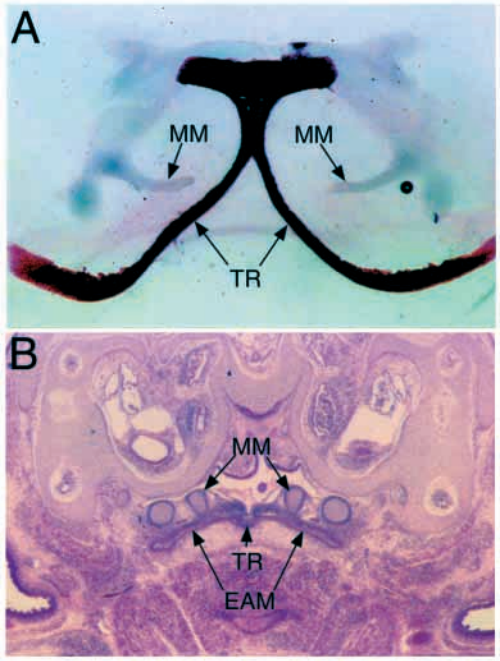

Fig. 6. Middle ear of E18.5 *Acvr2* mutant embryos. (A) Dissection of skeletal elements. The contralateral tympanic rings (TR) are fused in the midline. The manubrium of the malleus (MM) is fully developed and located in the plane defined by the tympanic ring. (B) Frontal sections through the ear region. The external acoustic meatus (EAM) is oriented towards the tympanic ring. The manubrium of the malleus is associated with its medial surface of the EAM.

Thus, despite the major malformations in this area of the *Acvr2* mutant mice, the tympanic ring, the EAM and the manubrium of the malleus are all present, and display a remarkable conservation of their normal anatomical relationships.

Altogether, the above results indicate that the EAM, tympanic ring and malleal manubrium are affected in a coordinated fashion, further supporting the existence of mechanisms coordinating the development of these structures.

Sox9 and *Prx1* induction by the EAM

The results presented so far suggest a developmental connection between the EAM and the manubrium of the malleus. However, they do not show the nature of this association. In the craniofacial region epithelia have been shown to be required for skeletogenesis in the underlying mesenchyme (Bee and Thorogood, 1980; Hall, 1980; Hall and Miyake, 1995). If a similar situation also occurs in the area of the developing ear, the EAM could then be playing an inductive role in the morphogenesis of the manubrium. This potential role of the EAM fits well with the results from the development of the middle ear ossicles in vitro shown in Fig. 2; however, they do not provide a proof of this possibility. For this, we decided to test the ability of the EAM to induce chondrogenesis on isolated first arch mesenchymes in vitro.

Theoretically, the most appropriate tissue for these studies would be the mesenchyme from the area where the manubrium develops shortly before this element is induced (i.e. E12.0-E12.25). However, there are no morphological references that allow a clean separation of this area from adjacent regions where other elements have already been induced (particularly that of the styloid process, see Fig. 3), which, when cultured in vitro without ectoderm, are able to proceed with chondrogenic differentiation (not shown). Therefore, at this stage it was not possible to isolate pieces of mesenchyme that allowed us to test reliably the EAM for potential inducing activities.

Earlier in development, however, skeletogenesis in the first arch mesenchyme has been reported to be dependent on epithelial signals (Hall, 1980). In control experiments we found that when intact E10.5 first branchial arches were cultured in vitro, *Sox9* expression (used as a marker for chondrogenic differentiation) could be detected in a central core of the arch after 2 days of incubation (Fig. 7A; Table 1). However, when the epithelium was removed prior to incubation, *Sox9* was either non-detectable or detected at low levels in a small peripheral area corresponding to the proximal portion of the first arch (Figs 7B and 8A; Table 1). Addition of first arch epithelium to the isolated mesenchyme restored *Sox9* activation (Fig. 7C; Table 1). These results indicated that *Sox9* expression in E10.5 first arch mesenchymes depends on interactions with the epithelium, so we decided to use this tissue in our experiments with the EAM.

Recombination experiments between first arch mesenchyme and EAM showed that this epithelium is able to induce mesenchymal expression of *Sox9* (Fig. 8C,E; Table 1). In all cases the induced *Sox9* expression was clearly stronger than

Table 1. *Sox9* and *Prx1* expression in mandibular mesenchymes in the presence and absence of epithelia

	Tissue cultured				
	Intact arch	Mesenchyme	Mesenchyme+1st arch epithelium	Mesenchyme+EAM distal	Mesenchyme+EAM proximal
Sox9	4/4	6/11*	5/6	6/9	2/9
Prx1	ND	4/4	ND	4/4	4/4

Values are the number of explants with detectable expression/the number of explants analyzed.
*When detected, the levels of *Sox9* expression were always low.
ND, not determined.

the basal *Sox9* domain sometimes found in the nonrecombined mesenchymes. However, the *Sox9*-inducing ability of the EAM had two peculiarities. First, although *Sox9* expression was associated with the EAM, it did not occur in direct contact with this epithelium (Fig. 8C,E). Instead, the area adjacent to the EAM was clearly negative for *Sox9*, which was expressed distal to this 'exclusion' area (Fig. 8C,E). This finding was interesting considering that in vivo the manubrium is not induced next to the EAM but deeper in the mesenchyme, at a distance from the surface ectoderm (Fig. 3). Second, the ability of the EAM to induce *Sox9* expression was clearer when it was recombined with the mesenchyme in areas corresponding to the proximal portion of the first arch than when it was placed in more distal areas (Fig. 8C,E). In the latter situation, clear induction was only seen in 2 out of 9 cases, and in those the *Sox9* domain was quite far from the EAM (Fig. 8C). These results indicate that the EAM has chondrogenic-inducing activity but some mechanisms must also exist to produce the observed spatial restriction of this activity.

Some data suggested that *Prx1* could play a role in generating the patterns of *Sox9* induction by the EAM in the first arch mesenchyme. First, *Prx1* is expressed in the mesenchyme attached to the EAM and downregulated in the area of the developing manubrium, where it shows a somewhat complementary expression pattern with *Sox9* (Fig. 9). Second, this gene is expressed in distal but not proximal areas of the mandibular arch at E10.5, and could then be involved in the differences of EAM activity along the proximal-distal axis (Cserjesi et al., 1992). Third, *Prx1* expression has been previously shown to depend on ectodermal signals (Kuratani et al., 1994). Therefore, it was possible that the EAM is able to induce mesenchymal expression of both *Prx1* and *Sox9* and that *Sox9* induction was possible only in *Prx1*-negative areas. To test this possibility we analyzed *Prx1* expression in recombination experiments similar to those described above. When the first arch mesenchyme was cultured in the absence of ectoderm, *Prx1* could be detected in distal but not proximal areas of the first arch mesenchyme (Fig. 8B). When the EAM was placed in distal areas of the first arch mesenchyme, *Prx1* induction was observed around the EAM (Fig. 8D). When the recombination was performed in the proximal area *Prx1* was also induced in the mesenchyme adjacent to the EAM (Fig. 8F). In this case, the *Prx1*-positive area was not very wide and was surrounded by a *Prx1*-free space which coincided with the *Sox9* expression domain. Altogether, these results indicate that the EAM is able both to induce chondrogenesis and to

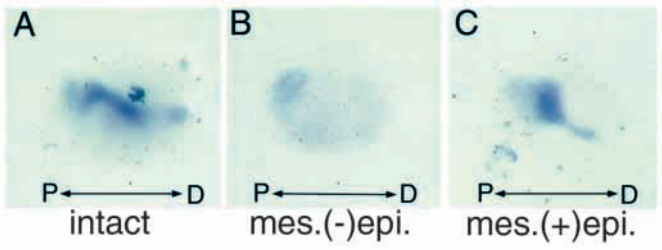

Fig. 7. *Sox9* induction in the first arch mesenchyme depends on the epithelium. (A) *Sox9* is induced in first branchial arches that have been incubated for 2 days without removal of the epithelium. (B) When the epithelium is removed from the mesenchyme before incubation, only low levels of *Sox9* expression are detected in the proximal area of the first branchial arch. (C) When epithelium and mesenchyme are first separated and recombined again before incubation, *Sox9* induction is restored. P and D indicate the proximal-distal axis in the incubated first arches.

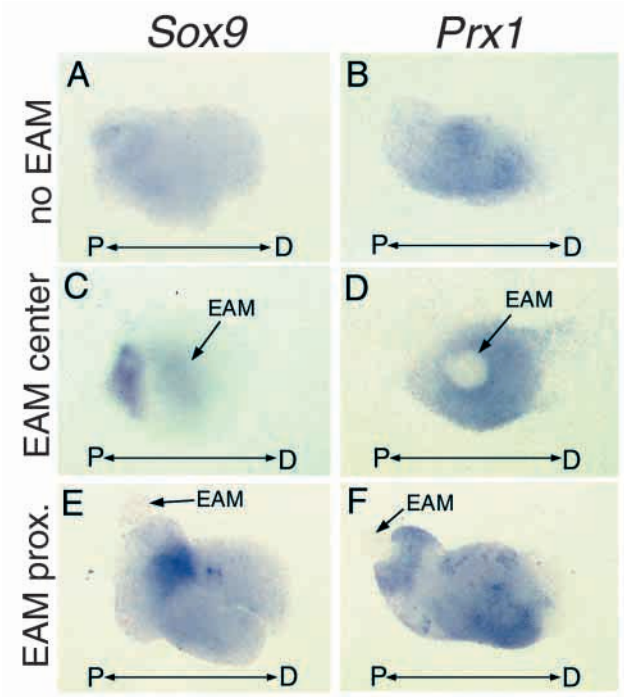

Fig. 8. Induction of *Prx1* and *Sox9* by the EAM. (A,B) After 2 days in culture, isolated E10.5 first arch mesenchymes show background levels of *Sox9* expression and moderate levels of *Prx1* expression in distal areas. (C,D) Recombination of isolated external acoustic meati (EAM) in central areas of the mesenchyme results in peripheral induction of *Sox9* in the proximal region, away from the EAM. *Prx1* is induced around the EAM but is still excluded from the proximal areas of the mesenchyme. (E,F) Recombination of EAM in proximal areas of the mesenchyme results in *Sox9* expression close to the EAM, but not in direct contact with it. *Prx1* is also induced in the proximal mesenchyme, in direct contact with the EAM. P and D indicate the proximal-distal axis in the incubated first arches.

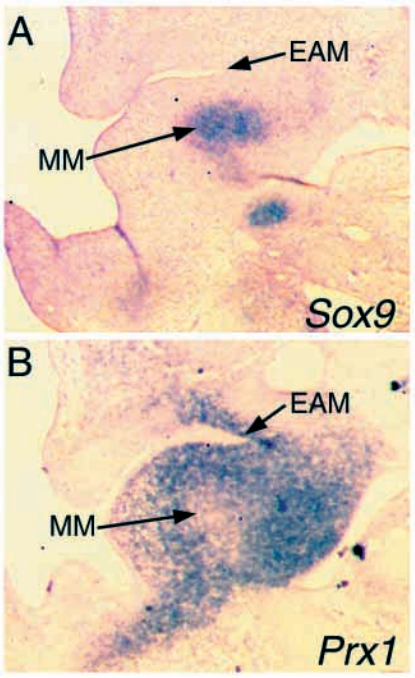

Fig. 9. Expression of *Sox9* and *Prx1* in the region of the developing manubrium. (A) *Sox9* is detected in the mesenchyme medial to the developing external acoustic meatus (EAM), in the region where the manubrium of the malleus (MM) is induced. (B) *Prx1* is expressed in the mesenchyme medial to the EAM in contact with the epithelia and downregulated in the area of the developing manubrium. *Sox9* and *Prx1* were detected by in situ hybridization on transverse sections of E13.5 mouse embryos. The sections are oriented with the rostral side to the top.

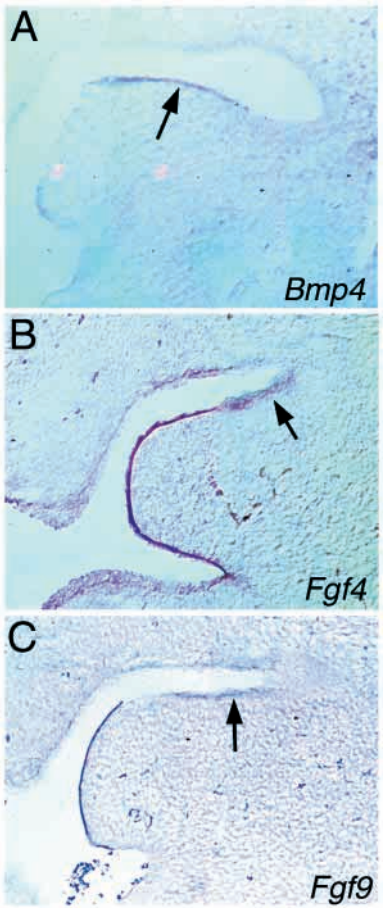

Fig. 10. Gene expression in the external acoustic meatus. Signals for *Bmp4* (A), *Fgf4* (B) and *Fgf9* (C) are detected in particular areas of the EAM (arrows) by in situ hybridization on transverse sections of E13.5 mouse embryos. The sections are oriented with the rostral side to the top.

delimitate where this is done, and that the latter probably requires the activation of *Prx1* expression.

Bmp4, *Fgf4* and *Fgf9* are expressed in the EAM

In an initial effort to determine the molecular nature of the signals mediating the activities of the EAM we looked for expression of some signaling molecules that have been implicated in skeletogenic processes or shown to be required at some step of craniofacial development. We found clear signals for *Bmp4*, *Fgf4* and *Fgf9* (Fig. 10). The expression of these genes is not extended throughout the EAM but seems to show some spatial restriction within the epithelium (Fig. 10; and not shown). We could not detect transcripts in the EAM for several other genes, including *Bmp5*, *Shh*, *Fgf8* and *Endothelin1* (not shown). The expression of two *FGFs* in the EAM is interesting considering that members of this gene family have been shown to upregulate *Sox9* expression in mouse primary chondrocytes and C3H10T1/2 cells (Murakami et al., 2000). These results indicate that the EAM is the source of factors that might be implicated in chondrogenesis, further supporting the results obtained in our recombination experiments.

DISCUSSION

In this study we have investigated the mechanisms governing development of the manubrium of the malleus. This structure, which develops from the mesenchyme of the proximal area of the first branchial arch, provides the connection between the tympanic membrane and the middle ear ossicle chain (Mallo, 1998). Therefore, it is of central importance for the functionality of the hearing apparatus. The first sign of manubrial induction can be detected at the caudal end of the developing malleus at early day 12 of mouse development. The induction of this element is completed about 1 day later, but full differentiation of cartilage structures still requires another day of development. It is also at this point in development when it starts to become integrated into the tympanic membrane as a consequence of the apposition of the ectodermal and endodermal epithelia of the first pharyngeal cleft and pouch, respectively (Mallo and Gridley, 1996).

EAM controls induction of the manubrium

In the craniofacial area, development of skeletal structures is controlled by a combination of patterning and signaling processes (Francis-West et al., 1998). The relative weight of each of these and their molecular nature varies from one region to another. In the case of the manubrium, positional

information in the mesenchyme seems not to be the main drive for its spatial and temporal patterns of development, based mainly on analysis of development of the middle ear ossicles in vitro. When the prospective ear region was dissected out and cultured under conditions that support endochondral differentiation, the manubrium developed only up to the extent to which it had been induced at the time of explantation. Therefore, it is probable that the mesenchyme at this stage does not contain a programe to complete manubrial development, but this programe is sequentially provided between E12.25 and E13.25. Similarly, in *Prx1* and *Gsc* null mutants, both of which show a selective deletion of the manubrium in otherwise quite normal mallei (Martin et al., 1995; Rivera-Pérez et al., 1995; Yamada et al., 1995), it is not straightforward to explain the absence of this element by considering direct patterning roles of these genes in the mesenchyme eventually contributing to manubrial development. In the case of *Gsc*, expression data and chimeric analyses could be interpreted to support such a direct role because *Gsc* is expressed in the developing manubrium and *Gsc* null cells are excluded from this structure (Rivera-Pérez et al., 1999). However, the same is also true for the rest of the malleus, which is not visibly affected by the null mutation (Rivera-Pérez et al., 1995, 1999; Yamada et al., 1995). In the case of the *Prx1* gene, transcription seems to be downregulated in the developing manubrium, as has been described for other developing endochondral elements (Cserjesi et al., 1992; Kuratani et al., 1994). These expression data do not provide any explanation for the quite selective negative effect of the *Prx1* mutation on manubrial development.

An alternative hypothesis, more consistent with our data, considers that development of the manubrium is controlled by the EAM. Accordingly, the EAM would be the origin of signals that induce chondrogenic differentiation in the underlying mesenchyme, eventually resulting in manubrial formation.

Our analyses of the spatial and temporal dynamics of EAM and manubrial development support this hypothesis. The EAM results from the invagination of the first pharyngeal cleft ectoderm (Carlson, 1994; Mallo, 1998). In the mouse, this process occurs between early day 12 and day 13 of development, following a semicircular motion from the first arch region into the second arch (Mallo, 1998). The induction of the manubrium also occurs following a similar temporal sequence, close to the medial surface of the EAM. Therefore, although other explanations are also possible, these results are consistent with and suggestive that the EAM plays a role in the induction of the manubrium.

This hypothesis also provides an explanation for the results of the in vitro ossicle development experiments and for the ear phenotype of *Gsc* and *Prx1* null mutant embryos. In the case of the cultured explants, culture conditions seemed not to allow further development of the EAM. If manubrial development depends on inducing signals from this epithelium, it is expected that manubrial induction becomes 'frozen' at the time of explantation. Thus, the level of development achieved by this skeletal structure would reflect the stage of induction at the time of dissection. In the case of the *Prx1* and *Gsc* mutants, EAMs cannot be detected in histological sections through the middle ears (Rivera-Pérez et al., 1995; Yamada et al., 1995; Fig. 4), though this is probably secondary to the absence of tympanic rings because neither of the genes, both encoding for transcription factors, is expressed in the epithelial structure (Mallo and Gridley, 1996; Rivera-Pérez et al., 1999; Fig. 9). If signals from the EAM are required for manubrial development, this structure would fail to develop in these embryos without any deleterious effect on the rest of the malleus.

The phenotypes resulting from RA teratogenesis are also consistent with the EAM playing a role in manubrial induction. Analysis of the different middle ear malformations obtained after RA treatment of pregnant females shows that the two structures are affected in a coordinated fashion. When an EAM was present, a cartilage resembling the manubrium was always present in the right relative position, irrespective of the morphology of the rest of the malleus; conversely, in the absence of EAM, a similar cartilage could not be identified. Although other explanations for these observations are possible (Mallo, 1997), they are also consistent with the manubrium requiring EAM-derived inducing signals for its development.

All the above-mentioned arguments support a role of the EAM in manubrial development, but they do not provide a direct proof of it. The strongest evidence in support of the inducing activity of the EAM comes from recombination studies between this epithelium and isolated first arch mesenchyme. Those experiments showed that the EAM is the source of an activity capable of inducing *Sox9* in the mesenchyme. However, this activity seems to be modulated by other EAM-dependent signals, since *Sox9* is only induced in the mesenchyme at a certain distance from the EAM. This property is interesting considering that in normal development the manubrium is not induced in direct contact with the EAM but deeper in the underlying mesenchyme. Therefore, the *Sox9*-inducing property of the EAM found in vitro resembles the physiological situation. It is possible that *Prx1* plays a role in this site-restricted chondrogenesis. In the embryo this gene is strongly expressed in the mesenchyme adjacent to the EAM, but downregulated in the area undergoing chondrogenesis. Similarly, in our recombination experiments, the EAM was able to induce *Prx1* expression in direct contact with the epithelium, and the *Sox9* and *Prx1* expression domains seemed complementary. However, whether Prx1, alone or in combination with other molecules, actually mediates antichondrogenic activities from the EAM cannot be determined from our results. Some support for this possibility comes from the spatial coincidence of the *Prx1* expression domain and the antichondrogenic activity. In addition, some of the phenotypic traits of the *Prx1* mutant mice (Martin et al., 1995) could be interpreted on the basis of such an activity. Those embryos display a variety of skeletogenic alterations, which have in common deficiencies of dermal bones and extra endochondral elements in different craniofacial areas. Interestingly, very few hypomorphic defects are detectable in endochondral elements of the craniofacial area of *Prx1* mutants, those in the malleal manubrium being the most salient, further suggesting an indirect mechanism for this phenotypic trait.

Nature of the signals

Our results suggest the existence of epithelial signals responsible for both the chondrogenic inducing and restricting activities. One possibility is that the different areas of *Sox9* and *Prx1* induction result from a double signaling system, one with longer diffusing ability that induces *Sox9* and another of shorter range but with dominant activity over the former that

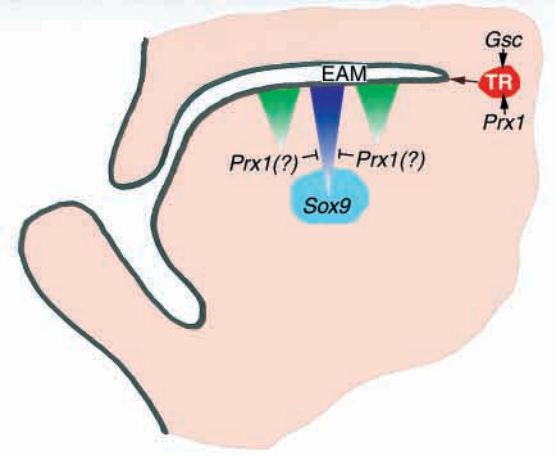

Fig. 11. Hypothetical model for the role of the external acoustic meatus in the morphogenesis of the tympanic membrane. The invagination of the external acoustic meatus (EAM) is controlled by the tympanic ring (TR). Formation of the TR is controlled by a variety of genes, including *Gsc* and *Prx1*. When the EAM invaginates, it becomes the source of signals with opposing activities. One of them (blue), probably mediated by *Sox9* activation, induces chondrogenesis; the other (green) represses cartilage formation. The latter activity acts on a shorter range but is dominant over the first. In addition, it is possible that the antichondrogenic function is mediated by the *Prx1* gene. The combined action of both activities results in the proper spatial and temporal induction of the malleal manubrium.

blocks chondrogenesis (Fig. 11). This model considers diffusible factors, but mechanisms requiring direct cellular contacts cannot be ruled out, particularly for the *Prx1* inducing activity. For instance, a cell relay system could mediate transmission of *Sox9* and/or *Prx1* inducing activities. Mixed mechanisms are also possible.

It is known that epithelial mesenchymal interactions are important for skeletogenesis in several facial areas (Bee and Thorogood, 1980; Hall, 1980; Hall and Miyake, 1995). However, the molecular nature of the signals involved is not so clear. A variety of data have demonstrated the requirement of secreted molecules like Fgf8, Shh, Bmp7 and Bmp5 or Endothelin 1 for development of the branchial arch area (Kurihara et al., 1994; Clouthier et al., 1998; Trumpp et al., 1999; Solloway and Robertson, 1999; Ahlgren and Bronner-Fraser, 1999; Hu and Helms, 1999). However, it is not clear whether any of these factors is directly involved in skeletogenic induction or if the observed skeletal defects are the consequence of alterations in growth and survival processes (Clouthier et al., 2000; Trumpp et al., 1999; Solloway and Robertson, 1999; Ahlgren and Bronner-Fraser, 1999; Hu and Helms, 1999). Our initial analyses revealed that *Bmp4*, *Fgf4* and *Fgf9* are expressed in the EAM. BMP and FGF signals have been shown to cooperate for positioning mesenchymal expression of *Pax9* in the tooth primordia (Neubüser et al., 1997). Interactions between these two signaling systems might also play a role in proper induction of the manubrium. The recent finding that FGFs are able to upregulate *Sox9* in mouse primary chondrocytes and C3H10T1/2 cells (Murakami et al., 2000) suggests that the chondrogenic-inducing processes might be mediated by the FGFs. However, the role that these and other factors actually play in vivo remains to be determined. Identification of further candidate molecules, combined with their functional evaluation, will be required to fully understand the molecular nature of these processes.

Coordinated development of the tympanic membrane

The morphogenesis of the eardrum implies coordinated development of elements of different embryological origins (Carlson, 1994; Mallo, 1998). Our data indicate that this coordination is achieved through sequential interactions between different components of the tympanic membrane, which mutually influence development of each other. Initially, the tympanic ring, of neural crest origin (Novacek, 1993), coordinates the invagination of the first pharyngeal cleft to form the EAM (Mallo and Gridley, 1996). This process ensures that the external auditory canal is located in the proper position in the mature temporal bone (Michaels and Soucek, 1989; Mallo and Gridley, 1996). As the EAM is being formed, it becomes the source of signals which, acting on the adjacent mesenchyme, coordinate formation of the manubrium. This process ensures that the manubrium develops in a position relative to the epithelia that allows its insertion into the mature tympanic membrane when the apposition of the medial surface of the EAM and the lateral endoderm of the middle ear cavity is completed. The high efficiency of this mechanism is revealed by the phenotype of *Acvr2* mutant embryos in which a fairly normal-looking tympanic area succeeded in developing at an ectopic location, in the absense of all other mandibular arch structures.

Extension of the model to other areas

Quite a common characteristic of skeletogenesis in different body areas is that skeletal elements are induced in the mesenchyme at a distance from the surface. For instance, Meckel's cartilage develops in a central core of the first branchial arch, not attached to the surface ectoderm (Miyake et al., 1996b). This is similar to what we have described for the manubrium. If similar principles are applied in these areas as in the region of the manubrium, it is possible that the proper spatial induction of those elements is also determined by complementary sets of signals from the ectoderm, as we have described for the region of the manubrium. Experiments are currently in progress in our laboratory to try to address this issue.

We thank Klaus Kratochwil for his detailed description of the procedure to perform the mesenchymal-epithelial recombination experiments, Gail Martin, Brigid Hogan and David Ornitz for probes, Stephan Gasca for having asked the right question, and Randy Cassada for reading the manuscript.

REFERENCES

Ahlgren, S. C. and Bronner-Fraser, M. (1999). Inhibition of Sonic hedgehog signaling *in vivo* results in craniofacial neural crest cell death. *Curr. Biol.* **9,** 1304-1314.

Bee, J. and Thorogood, P. (1980). The role of tissue interactions in the skeletogenic differentiation of avian neural crest cells. *Dev. Biol.* **78,** 47-62.

Carlson, B. M. (1994). *Human Embryology and Developmental Biology*. Mosby-Year Book, Inc., St Louis.

Clouthier, D. E., Hosoda, K., Richardson, J. A., Williams, S. C.,

Yanagisawa, H., Kuwaki, T., Kumada, M., Hammer, R. E. and Yanagisawa, M. (1998). Cranial and cardiac neural crest defects in endothelin-A receptor-deficient mice. *Development* **125,** 813-824.

Clouthier, D. E., Williams, S. C., Yanagisawa, H., Wieduwilt, M., Richardson, J. A. and Yanagisawa, M. (2000). Signaling pathways crucial for craniofacial development revealed by endothelin-A receptor-deficient mice. *Dev. Biol.* **217,** 10-24.

Colvin, J. S., Feldman, B., Nadeau, J. H., Goldfarb, M. and Ornitz, D. M. (1999). Genomic organization and embryonic expression of the mouse *fibroblast growth factor 9* gene. *Dev. Dyn.* **216,** 72-88.

Couly, G. F., Coltey, P. M. and Le Douarin, N. M. (1993) The triple origin of skull in higher vertebrates: a study in quail-chick chimeras. *Development* **117,** 409-429.

Cserjesi, P., Lilly, B., Bryson, L., Wang, Y., Sassoon, D. A. and Olson, E. N. (1992). MHox: a mesodermally restricted homeodomain protein that binds an essential site in the muscle creatine kinase enhancer. *Development* **115,** 1087-1101.

Francis-West, P., Ladher, R., Barlow, A. and Graveson, A. (1998). Signalling interactions during facial development. *Mech. Dev.* **75,** 3-28.

Hall, B. K. (1980). Tissue interactions and the initiation of osteogenesis and chondrogenesis in the neural crest-derived mandibular skeleton of the embryonic mouse as seen in isolated murine tissues and in recombinations of murine and avian tissues. *J. Embryol. exp. Morph.* **58,** 251-264.

Hall, B. K. and Miyake, T. (1995). Divide, accumulate, differentiate: cell condensation in skeletal development revisited. *Int. J. Dev. Biol.* **39,** 881-893.

Hu, D. and Helms, J. A. (1999). The role of Sonic hedgehog in normal and abnormal craniofacial morphogenesis. *Development* **126,** 4873-4884.

Jones, C. M., Lyons, K. M. and Hogan, B. L. M. (1991). Involvement of *Bone Morphogenetic Protein-4* (BMP-4) and *Vgr-1* in morphogenesis and neurogenesis in the mouse. *Development* **111,** 531-542.

Kanzler, B., Kuschert, S. J., Liu, Y.-H. and Mallo, M. (1998). *Hoxa2* restricts the chondrogenic domain and inhibits bone formation during development of the branchial area. *Development* **125,** 2587-2597.

Köntges, G. and Lumsden, A. (1996). Rhomboencephalic neural crest segmentation is preserved throughout craniofacial ontogeny. *Development* **122,** 3229-3242.

Kuratani, S., Martin, J. F., Wawersik, S. Lilly, B., Eichele, G. and Olson, E. N. (1994). The expression pattern of the chick homeobox gene *gMHox* suggests a role in patterning of the limbs and face and in compartimentalization of the somites. *Dev. Biol.* **161,** 357-369.

Kurihara, Y., Kurihara, H., Suzuki, H., Kodama, T., Maemura, K., Nagai, R., Oda, H., Kuwaki, T., Cao, W.-H., Kamada, N., Jishage, K., Ouchi, Y., Azuma, S., Toyoda, Y., Ishikawa, T., Kumada, M. and Yazaki, Y. (1994). Elevated blood pressure and craniofacial abnormalities in mice deficient in *endothelin-1*. *Nature* **368,** 703-710.

Mallo, M. (1997). Retinoic acid disturbs mouse middle ear development in a stage-specific fashion. *Dev. Biol.* **184,** 175-186.

Mallo, M. (1998). Embryological and genetic aspects of middle ear development. *Int. J. Dev. Biol.* **42,** 11-22.

Mallo, M. and Gridley, T. (1996). Development of the mammalian ear: coordinate regulation of formation of the tympanic ring and the external acoustic meatus. *Development* **122,** 173-179.

Mallo, M. and Brändlin, I. (1997). Segmental identity can change independently in the hindbrain and rhombencephalic neural crest. *Dev. Dyn.* **210,** 146-156.

Martin, J. F., Bradley, A. and Olson, E. N. (1995). The *paired*-like homeo box gene *Mhox* is required for early events of skeletogenesis in multiple lineages. *Genes Dev.* **9,** 1237-1249.

Matzuk, M. M., Kumar, T. R. and Bradley, A. (1995). Different phenotypes for mice deficient in either activins or activin receptor type II. *Nature* **374,** 356-360.

Michaels, L. and Soucek, S. (1989). Development of the stratified squamous epithelium of the human tympanic membrane and external canal: the origin of auditory epithelial migration. *Am. J. Anat.* **184,** 334-344.

Miyake, T., Cameron, A. M. and Hall, B. K. (1996a). Detailed staging of inbred C57Bl/6 mice between Theiler's [1972] stages 18 and 21 (11-13 days of gestation) based on craniofacial development. *J. Craniofac. Genet. Dev. Biol.* **16,** 1-31.

Miyake, T., Cameron, A. M. and Hall, B. K. (1996b). Stage-specific onset of condensation and matrix deposition for Meckel's and other first arch cartilages in inbred C57BL/6 mice. *J. Craniofac. Genet. Dev. Biol.* **16,** 32-47.

Murakami, S., Kan, M., McKeehan, W. L. and de Crombrugghe, B. (2000). Up-regulation of the chondrogenic *Sox9* gene by fibroblast growth factors is mediated by the mitogen-activated protein kinase pathway. *Proc. Natl. Acad. Sci. USA* **97,** 1113-1118.

Neubüser, A., Peters, H., Balling, R. and Martin, G. R. (1997). Antagonistic interactions between FGF and BMP signaling pathways: A mechanism for positioning the sites of tooth formation. *Cell* **90,** 247-255.

Niswander, L. and Martin, G. R. (1992). *Fgf-4* expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development* **114,** 755-768.

Noden, D. M. (1988). Interactions and fates of avian craniofacial mesenchyme. *Development* **103 Supplement**, 121-140.

Novacek, M. J. (1993). Patterns of diversity in the mammalian skull. In *The Skull*, vol. 2 (ed. J. Hanken and B. K. Hall), pp. 438-545. University of Chicago Press, Chicago.

Rivera-Pérez, J., Mallo, M., Gendron-Maguire, M., Gridley, T. and Behringer, R. R. (1995). *Goosecoid* is not an essential component of the mouse gastrula organizer but is required for craniofacial and rib development. *Development* **121,** 3005-3012.

Rivera-Pérez, J. A., Wakamiya, M. and Behringer, R. R. (1999). *Goosecoid* acts cell autonomously in mesenchyme-derived tissues during craniofacial development. *Development* **126,** 3811-3821.

Solloway, M. J. and Robertson, E. J. (1999). Early embryonic lethality in *Bmp5;Bmp7* double mutant mice suggests functional redundancy within the 60A subgroup. *Development* **126,** 1753-1768.

Song, J., Oh, S. P., Schrewe, H., Normura, M., Lei, H., Okano, M., Gridley, T. and Li, E. (1999). The type II activin receptors are essential for egg cylinder growth, gastrulation and rostral head development in mice. *Dev. Biol.* **213,** 157-169.

Trumpp, A., Depew, M. J., Rubenstein, J. L. R., Bishop, J. M. and Martin, G. R. (1999). Cre-mediated gene inactivation demonstrates that FGF8 is required for cell survival and patterning of the first branchial arch. *Genes Dev.* **13,** 3136-3148.

Wright, E., Hargrave, M. R., Christiansen, J., Cooper, L., Kun, J., Evans, T., Gangadharan, U., Greenfield, A. and Koopman, P. (1995). The *Sry*-related gene *Sox9* is expressed during chondrogenesis in mouse embryos. *Nature Genetics* **9,** 15-20.

Yamada, G., Mansouri, A., Torres, M., Stuart, E. T., Blum, M., Schultz, M., De Robertis, E. M. and Gruss, P. (1995). Targeted mutation of the murine *goosecoid* gene results in craniofacial defects and neonatal death. *Development* **121,** 2917-2922.

Zhu, C. C. Yamada, G. and Blum, M. (1997). Correlation between loss of middle ear bones and altered *goosecoid* gene expression in the branchial region following retinoic acid treatment of mouse embryos in vivo. *Biochem. Biophys. Res. Commun.* **235,** 748-753.