Bapx1 regulates patterning in the middle ear: altered regulatory role in the transition from the proximal jaw during vertebrate evolution

Abigail S. Tucker¹*, Robert P. Watson²*, Laura A. Lettice², Gen Yamada³ and Robert E. Hill²†

¹Department of Craniofacial Development and Orthodontics, Kings College London, Floor 28, Guy’s Tower, Guy’s Hospital, London SE1 9RT, UK
²MRC Human Genetics Unit, Western General Hospital, Crewe Rd, Edinburgh EH4 2XU, UK
³Center for Animal Resources and Development (CARD), Kumamoto University, Kumamoto, Japan

*These authors have contributed equally to the work
†Author for correspondence (e-mail: robert.hill@hgu.mrc.ac.uk)

Accepted 2 December 2003

Summary

The middle ear apparatus is composed of three endochondrial ossicles (the stapes, incus and malleus) and two membranous bones, the tympanic ring and the gonium, which act as structural components to anchor the ossicles to the skull. Except for the stapes, these skeletal elements are unique to mammals and are derived from the first and second branchial arches. We show that, in combination with goosecoid (Gsc), the Bapx1 gene defines the structural components of the murine middle ear.

During embryogenesis, Bapx1 is expressed in a discrete domain within the mandibular component of the first branchial arch and later in the primordia of middle ear-associated bones, the gonium and tympanic ring. Consistent with the expression pattern of Bapx1, mouse embryos deficient for Bapx1 lack a gonium and display hypoplasia of the anterior end of the tympanic ring. At E10.5, expression of Bapx1 partially overlaps that of Gsc and although Gsc is required for development of the entire tympanic ring, the role of Bapx1 is restricted to the specification of the gonium and the anterior tympanic ring. Thus, simple overlapping expression of these two genes appears to account for the patterning of the elements that compose the structural components of the middle ear and suggests that they act in concert.

In addition, Bapx1 is expressed both within and surrounding the incus and the malleus. Examination of the malleus shows that the width, but not the length, of this ossicle is decreased in the mutant mice. In non-mammalian jawed vertebrates, the bones homologous to the mammalian middle ear ossicles compose the proximal jaw bones that form the jaw articulation (primary jaw joint). In fish, Bapx1 is responsible for the formation of the joint between the quadrate and articular (homologues of the malleus and incus, respectively) enabling an evolutionary comparison of the role of a regulatory gene in the transition of the proximal jawbones to middle ear ossicles. Contrary to expectations, murine Bapx1 does not affect the articulation of the malleus and incus. We show that this change in role of Bapx1 following the transition to the mammalian ossicle configuration is not due to a change in expression pattern but results from an inability to regulate Gdf5 and Gdf6, two genes predicted to be essential in joint formation.

Movies available online

Key words: Bapx1, Middle ear, Tympanic ring, Gonium, goosecoid, Evolution, Mouse

Introduction

During the course of evolution a remarkable morphological transformation gave rise to the ossicles of the mammalian middle ear. The primary jaw articulation of nonmammalian vertebrates was replaced in mammals by a secondary articulation formed by the squamosal and the dentary (reviewed by Fleischer, 1978; Allin and Hopson, 1992). Comparative anatomy, embryology and paleontology suggest that the primary articulation, along with an adjacent bone, the hyomandibula (columella in chick and reptile), were subsequently incorporated into the mammalian middle ear such that the articular, quadrate and hyomandibula are equivalent to the malleus, incus and stapes, respectively (Reichert, 1837). The mammalian middle ear apparatus, which is derived from the first and second branchial arches, is generally described as being composed of these three ossicles. However, the middle ear also includes two structural components, the tympanic ring and the gonium, that anchor these bones to the skull. Both are generated by intramembranous ossification and correspond to the angular and the prearticular of non-mammalian vertebrates (Gaupp, 1911; Voit, 1924).

Current conjecture on the molecular changes that accompany evolution is centred on changes within regulatory proteins. Analysis of molecular changes that are crucial for morphological transitions are feasible in vertebrates as integral changes in morphology that characterise a class are well documented. The homeobox-containing transcription factors have been implicated as key elements in regulatory changes and the data increasingly supports a role for both single homeobox genes and whole complex Hox clusters in the
evolution of animal morphology (e.g. Grenier and Carroll, 2000; Grandien and Sommer, 2001). Bapx1 (Nkx3.2) is an evolutionarily conserved homeobox-containing gene that belongs to the NK-2 family of transcription factors (Kim and Nirenberg, 1989; Tribioli et al., 1997; Tribioli and Lufkin, 1997) and is most closely related to the Drosophila bagpipe (Nkx5) gene. In vertebrates, craniofacial expression of the gene is detected (Newman et al., 1997), including a large region of the intermediate first arch that encompasses the jaw joint region. Recent work carried out by Miller et al. (Miller et al., 2003) reveals a role for the gene in regulating the patterning of the zebrafish (D. rerio) jaw joint. In this organism, Bapx1 is initially expressed in the mesenchyme of the mandibular arch primordia but later can be detected in the cells within and surrounding the jaw joint. Downregulation of the gene, using Bapx1-specific morpholinos, results in a dose-dependent loss of the jaw joint, and is characterised by a fusion between the constituent cartilages, the quadrate and articular.

Given the evolutionary relationship between the jaw bones of fish and the middle ear bones of mammals, these observations raise the possibility that Bapx1 may play a crucial role in regulating the development of the murine middle ear. Consistent with this hypothesis, expression of the gene has been reported at E10.5 in the mandibular portion of the first branchial arch, and later in precursor of Meckel’s cartilage (Tribioli et al., 1997). Previous analyses of mice carrying targeted mutations in the Bapx1 gene have reported a role for the gene in regulating the development of the axial skeleton, spleen and the gastrodudodenal region of the gut (Lettice et al., 1999; Tribioli and Lufkin, 1999; Akazawa et al., 2000). We show here that Bapx1 plays a crucial role in regulating the development of the structural elements of the murine middle ear, and provide evidence to suggest that it does so in combination with Gsc, a gene with a well-characterised role in tympanic ring development. Furthermore, we demonstrate that the role of Bapx1 in development of the middle ear ossicles is restricted to regulating the width of the malleus and that the lack of the predicted malleal/incal fusions in Bapx1–/– mice results from regulatory changes in genes involved in joint formation.

Materials and methods

Embryos

Bapx1 was inactivated by the insertion of a pMC1neo-polyA (Stratagene) cassette into the 3’ end of exon 1 (Lettice et al., 1999). Postimplantation mouse embryos were collected at the desired stages, considering the day of the vaginal plug as E0.5, and were genotyped as described (Lettice et al., 1999). Chick embryos were collected at the desired ages following the staging system of Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Probes and in situ hybridisation

DIG in situ hybridisation was performed on whole-mount embryonic day (E) 10.5-12.5 mouse embryos and on sections derived from paraformaldehyde-fixed and paraffin-embedded tissue essentially as described by Wilkinson (Wilkinson, 1992). Radioactive 35S in situ hybridisation procedures were carried out as described by Tucker et al. (Tucker et al., 1999). Following skeletal staining and conventional imaging, samples were embedded in 2% LMP agarose made up in 80% glycerol and analysed by OPT essentially as described (Sharpe et al., 2002). Because the samples had been cleared previously in KOH/glycerol, the methanol and BABB pre-scan procedures were omitted and the scans were performed in a cuvette filled with 80% glycerol.

Skeletal staining of mouse and chick embryos

E14.5 to postnatal day 2 (P2) mouse embryos were stained for bone and cartilage formation using the method described by Kessel and Gruss (Kessel and Gruss, 1991). E7 to E14 chick embryos were decapitated and heads fixed in 4% PFA overnight. Heads were then washed in PBS and stained in Alcian Blue 8GX overnight (5% of a 0.1% Alcian Blue solution, 5% acetic acid in ethanol). Stained heads were rehydrated through an ethanol series over several days and then cleared in 1% KOH. For staining of cranial bones, alcin blue heads were stained in 1% Alizarin Red in 0.5% KOH overnight. Heads were washed in 0.5% KOH to remove excess stain. Once cleared, heads were taken up through a glycerol series for storage.

Statistical analysis

Middle ears were dissected from E18.5 wild-type (n=10), heterozygous (n=13) and homozygous (n=8) mice. The dimensions of the malleus were measured and pairwise Student’s t-tests were carried out on the data generated.

Results

Bapx1 is required for tympanic ring and gonium development and regulates the width of the malleus

Skeletal preparations of E18.5 and P2 mouse middle ears (Figs 1-3) were examined for possible defects in the articulation of the malleus and incus due to the loss of Bapx1. Contrary to reports in zebrafish (Miller et al., 2003), the malleal/incal joint was unaltered in Bapx1–/– embryos (Fig. 1A,B). Furthermore, development of the middle ear ossicles appeared to be largely unaffected by the loss of the Bapx1 gene, with the incus and stapes developing as normal with respect to both size and articulation (Fig. 1A,B and data not shown). However, although the processus brevis and manubrium of the malleus develop normally in Bapx1 mice, the width of this ossicle is significantly reduced in both heterozygotes and homozygotes (P<0.0002 and P<0.00001 respectively) (Fig. 1C). Other dimensions of the malleus, including its length (Fig. 1D), remain unaltered (wild type versus heterozygotes P=0.85; wild type versus homozygotes P=0.95). Further examination of the middle ear revealed that the structural components of the middle ear are also affected (Fig. 2A-F). Bapx1–/– mice displayed defects in both the gonium and tympanic ring. The gonium is an investing bone that lies on the surface of the malleus. After birth, the gonium invades the malleus and becomes the process folii (anterior or gracilis) of the mature malleus at the point where the malleus separates from Meckel’s cartilage. Hence, the mature malleus is a composite bone with endochondrial and membraneous components (DePew et al., 2002). The tympanic or ectotympanic underlies the malleus in a similar position to the gonium. The reflected lamina process back caudally as a ring towards the styloid process, thus delimiting the tympanic membrane. In Bapx1–/– mice, the tympanic ring is hypoplastic, with development of the characteristic anterior thickening of this structure completely absent (compare Fig. 2A with 2E and see Movies 1 and 2 at http://dev.biologists.org/supplemental). Furthermore, the gonium, which normally develops adjacent to this region of the tympanic ring and Meckel’s cartilage, is absent (compare Fig.
Bapx1 regulates patterning in the middle ear

2B with 2F and Movies 1 and 2 at http://dev.biologists.org/supplemental). Development of the middle ear components continues after birth (Huangff and Saunders, 1983). By P2 the gonium has fused to the malleus and the process is completed with the subsequent fusion of these bones to the tympanic. Bapx1–/– mice die perinatally and so it is not possible to investigate the effects of loss of Bapx1 on postnatal middle ear development. Bapx1+/– mice, however, are viable and analysis of middle ears in P2 neonates is shown in Fig. 3. Although the extent of gonium hypoplasia in these animals is variable (data not shown), in all animals examined to date (n=7) the gonium rudiment shows signs of fusion with the malleus (Fig. 3C,D). Furthermore, comparative analyses of adult wild-type and Bapx1+/– middle ears reveals no overt phenotype associated with the earlier observed gonium hypoplasia, with the malleus, gonion and tympanic forming a continuous skeletal structure (data not shown).

Bapx1 expression correlates with tympanic ring condensation and development of the gonion

Bapx1 expression has been previously reported in the inferior face of the mandibular portion of the first branchial arch at E10.5, and later, at E12.5, in a domain associated with Meckel’s cartilage (Tribioli et al., 1997) (Fig. 4A,B). Our observations support these findings but also reveal expression of Bapx1 at E12.5 in the mesenchymal condensation that ultimately gives rise to the tympanic ring (Mallo and Grindley, 1996) (Fig. 4C,D). Strong expression of Bapx1 is also observed in a small mesenchymal condensation medial to the developing tympanic ring and adjacent to Meckel’s cartilage that we postulate to be the prospective gonium (Fig. 4D).

Later in gestation, at E15.5, Bapx1 expression is particularly high in the periphery of the malleus and incus, in the region where these two ossicles articulate (Fig. 4G,H). Outside this region, Bapx1 expression is detected throughout the ossicles (Fig. 4E,F). By this stage, the tympanic ring has started to condense between Meckel’s cartilage and the external auditory (acoustic) meatus (EAM). Bapx1 expression extends from Meckel’s cartilage out towards the anterior (rostral) part of the tympanic but is not expressed within the condensation (Fig. 4I,J). Bapx1 expression is observed at E15.5 within the gonial bone condensation that forms next to the proximal region of Meckel’s cartilage (Fig. 4I,J). Bapx1 expression is also detected in the cells of the periphery of the proximal part of Meckel’s cartilage (Fig. 4I). Expression is also observed
around the most caudal part of the EAM (Fig. 4K,L). By E17.5, the gonium has also started to ossify. At this stage, expression of Bapx1 is still observed around the gonium and tympanic ring, but not in the bones themselves (Fig. 4M,N). Expression of Bapx1 in the malleus and incus has reduced but remains high at the site of their articulation by these late stages (Fig. 4O,P).

**Bapx1 and Gsc function independently to specify the tympanic ring**

Mouse studies have revealed numerous genes that, when inactivated, affect the development of the bones associated with the middle ear including Fgf8 (Trumpp et al., 1999), Prx1 (Martin et al., 1995) and Msx1 (Satokata and Maas, 1994). Expression of each of these genes is unaffected at E10.5 in Bapx1+/− mice (data not shown). Of those genes that give rise to phenotypes that resemble those observed in Bapx1+/- mice, Gsc is the best characterised. Gsc is expressed at E10.5 in the mandibular component of the first branchial arch and later in the region of the developing auditory meatus and malleus (Gaunt et al., 1993). Accordingly, inactivation of the gene results in a variety of defects associated with the middle ear that include aplasia of the tympanic cavity and the external auditory meatus and defects in the manubrium and processus brevis of the malleus. Significantly, Gsc−/− mice lack a tympanic ring (Rivera-Pérez et al., 1995; Yamada et al., 1995) or display only a rudimentary element (Kuratani et al., 1999), and are reported to exhibit mild hypoplasia of the gonial bone (Rivera-Pérez et al., 1995; Yamada et al., 1995). Given the phenotypic similarities of the Bapx1 and Gsc knockout mice, the possibility that the two genes might function together to specify tympanic ring development was investigated. At E10.5, both genes are expressed in most, perhaps all, cells within well-defined domains within the mandibular component of the first branchial arch (Fig. 5A,B). Two-colour in situ hybridisation analysis reveals that these domains overlap within a small area in the caudoproximal region of the arch (Fig. 5C,D). Later in development at E15.5, Gsc is expressed strongly throughout the condensing tympanic (Fig. 5E,G,J,L) and around the EAM up to the malleus (Fig. 5L), coinciding with Bapx1 expression in the caudal region of the EAM (Fig. 5K) and in the anterior tympanic ring (Fig. 5E-G). Gsc expression is only weakly seen in the developing gonium, where Bapx1 is strongly expressed (Fig. 5F,G).

However, subsequent analyses of Gsc expression in Bapx1−/− embryos, together with the results obtained from the reciprocal experiment, suggest that these genes function independently of one another in the regulation of tympanic ring development. Unaltered Bapx1 expression is detectable in Gsc−/− embryos (Fig. 5H,I,M,N). Expression of Bapx1 is not only retained around Meckel’s cartilage, where the gonium can be seen condensing and where the anterior part of the tympanic would normally develop (Fig. 5H,I), but also is seen as a patch at the base of the malleus where the EAM would normally form (Fig. 5M,N). Similarly, Gsc expression in the first branchial arch is unaltered in Bapx1−/− mice at E10.5 (Fig. 5O,P) and persists in its wild-type expression domain later at E15.5 (Fig. 5Q,R).

**Comparison of tympanic ring and gonium development in mouse with prearticular and jaw development in chick**

The mammalian tympanic ring is thought to be homologous to the angular of non-mammalian gnathostomes, while the gonium is homologous to the prearticular (Gaupp, 1911; Voit, 1924). These two membranous bones are closely associated with the cartilaginous articular, which (according to Reichert’s theory) is homologous to the mammalian malleus (Reichert, 1837). An investigation of the development of these two membranous bones in species that represent the archetypal configuration of facial bones following (Fig. 6A-C), and preceding (Fig. 6D-F), the transition to the middle ear was undertaken.

In mice, development of the malleus at the proximal end of Meckel’s cartilage is evident by E14.5, although Alizarin Red staining reveals no sign of ossification at this stage (Fig. 6A). By E16.5 and E18.5, ossification of the tympanic ring and gonium, respectively, are detectable at the base of the malleus (Fig. 6B,C). From Alcian Blue staining of developing cartilage elements in the chick jaw, the quadrate and Meckel’s cartilage appear as two distinct sites of chondrogenesis at E7 (Fig. 6D). However, staining with type II collagen shows that initially these two elements are derived from a single precartilage condensation, the joint developing within this condensation as a region of cells that do not later chondrify (Wilson and Tucker, 2004). Similar observations have been described in the zebrafish (Schilling and Kimmel, 1997). Then at E9, the membranous bones start to ossify around the cartilaginous jaw joint, as indicated by staining for alizarin red (Fig. 6E). These include the angular that develops as a thin strip under the articular and Meckel’s cartilage, and the larger surangular (supra-angular)
that forms above these cartilages (data not shown). The surangular is thought to be homologous to the accessory malleus, a small bone lying above the anterior process of the malleus, seen in a few mammals but not in the mouse (Henson, 1974). The prearticular does not ossify until E11, when it appears as a shallow group of cells on the medial surface of the cartilaginous articular, next to the angular. This site of ossification is clearly formed by E13 (Fig. 6F). In some texts, the prearticular is described as an endochondral bone but this is clearly not the case as can be seen in sections (Fig. 7H) (Romanoff, 1960). The basis for regarding the prearticular as homologous to the gonium is due to the fact that the chorda tympani nerve runs forward between it and Meckel’s, a similar route being followed between Meckel’s and the gonium in mammals (de Beer, 1937; Goodrich, 1986). In addition, in birds the prearticular fuses with the articular (Voit, 1924), in the same way as the gonium fuses with the malleus in mammals.

If the prearticular and angular are homologous to the gonium and tympanic, Bapx1 expression is to be expected in the primordia of these membranous bones in the chick. At E7, as the cartilages have started to differentiate, strong expression of Bapx1 is observed in the quadrate and articular part of Meckel’s cartilage, and in the joint region itself, thus agreeing with the expression pattern described in zebrafish (Miller et al., 2003) (Fig. 7A,B). By E9, expression is downregulated in the cartilages themselves but remains high in the joint region (Fig. 7C,D). At this stage, expression is also seen around the developing membranous bones, the angular and surangular (Fig. 7E,F). The site of the presumptive prearticular was confirmed by using an early bone marker, Runx2. Runx2 is a marker of both developing bone and cartilage, and mutant mice lack both intramembranous and endochondrial ossification (Ducy et al., 1997; Komori et al., 1997). At E9, Runx2 shows a patch of expression on the medial side of the articular above the angular, although no condensation

**Fig. 4.** Expression of Bapx1 in the mouse middle ear. (A) Expression of Bapx1 during middle ear development can first be detected at E10.5, with expression of the gene in a discrete domain in the first branchial arch (blue arrowhead). (B) By E12.5, Bapx1 expression can be detected in the region of the condensing middle ear ossicles. (C,D) Closer inspection of this expression pattern reveals Bapx1 expression around Meckel’s cartilage (defined in C by expression of α-collagen) and in condensations that correspond to the condensing tympanic ring (yellow arrowhead) and gonium (black arrowhead). (E-P) Analysis of Bapx1 expression during late embryogenesis through sagittal sections stained with either Alcian Blue and chlorantine Fast Red (E,G,IK,M,O) or by 35S radioactive in situ hybridisation (F,H,J,L,N,P). (F,H,J,L) Dark-field photographs, Bapx1 expression shown by white silver grains. (N,P) Bright-field photos, Bapx1 expression shown by black grains. (E-J) E15.5, (K,L) E14.5, (M-P) E17.5. (E,F) Expression of Bapx1 both within and between (arrow) the malleus and incus. (G,H) Expression surrounding the malleus and incus in the incudo-malleal joint region. (I,J) Expression in the developing gonium (arrow) and around Meckel’s cartilage, but not within the differentiating tympanic ring. (K,L) Expression under the EAM (arrow). (M,N) Expression around the developing gonium (arrow) and tympanic. (O,P) Expression in the incudo-malleal joint (arrow). Me, Meckel’s cartilage; M, malleus; I, incus; S, stapes; Ty, tympanic ring; G, gonium; EAM, external auditory (acoustic) meatus.
is obvious at this site at this stage (Fig. 7E,G). Bapx1 can be seen to be expressed in a similar domain (Fig. 7F). By E12, the prearticular has started to ossify, and Bapx1 expression is limited to the cells surrounding the membraneous bones, rather than in the bones themselves (Fig. 7H,I). The expression pattern of Bapx1 in the angular and prearticular, therefore closely follows that of the tympanic and gonium with respect to relative timing and pattern.

Expression of Eya1, Gdf5 and Gdf6 in developing mouse middle ear

The lack of a phenotype between the malleus and incus of Bapx1−/− mice, as predicted by the zebrafish work and the conserved expression pattern, was unexpected. This might be due to compensation by the other member of the bagpipe family, Nkx3.1. Double knockouts for Bapx1 and Nkx3.1 show an enhanced skeletal phenotype compared to single Bapx1 mutants, demonstrating that the two genes do collaborate in some aspects of embryonic development (Herbrand et al., 2002). Nkx3.1 is expressed in epithelial structures of the face, such as the tongue and teeth but not the middle ear (Tanaka et al., 1999). Expression of Gsc is unaffected by the loss of Bapx1 expression within the first branchial arch. At E15.5 expression of Gsc is seen around Meckel’s cartilage (Me) and in the pharyngeal cleft between the first and second branchial arches (arrowhead). EAM, external auditory meatus; G, gonium; M, malleus; Me, Meckel’s cartilage; Ty, tympanic ring.

Fig. 5. Analysis of the relationship between Bapx1 and Gsc in murine middle ear development. (A–C) Whole-mount in situ hybridisation analysis of Bapx1 (A), Gsc (B) and Bapx1/Gsc (C) expression in E10.5 branchial arches. At E10.5, Bapx1 is expressed in two discreet domains within the mandibular component of the first branchial arch, including both the neural crest-derived mesenchyme (white arrowhead) and a small region of the branchial arch epithelium (yellow arrowhead). Gsc expression is restricted to the neural crest derived mesenchyme in the caudal half of the first branchial arch. (D) Schematic representation of the two expression domains highlighting the area of overlap in the caudoproximal region of the mandibular component of the first branchial arch. (E–N) In situ hybridisation analysis of Bapx1 expression in wild-type and Gsc−/− E15.5 embryos. (E–G,J,L) Sagittal sections through the middle ear region of an E15.5 mouse embryo. Stained with Alcian Blue and chloramine Fast Red (E,J), and by 35S radioactive in situ for Bapx1 (F,K) and Gsc (G,L). Bapx1 is expressed around and within the developing malleus (depending on the level of section within this ossicle) and coincides with Gsc expression in the anterior part of the tympanic ring (yellow arrows) (F,G). Bapx1 expression overlaps Gsc in the caudal mesenchyme above the developing EAM (red arrows) (K,L). High levels of Bapx1 are observed within and around the gonial (F), where only very weak expression of Gsc is observed (G). Unlike Gsc, which is expressed throughout the tympanic ring (yellow arrows in G,L), Bapx1 is expressed only around the anterior part of the tympanic (F,K). (H,I,M,N) Expression in Gsc+. The expression pattern of Bapx1 is maintained around and in the gonium and where the tympanic ring would normally form (H,I). Expression is also maintained under the malleus (red arrow), despite failure of the EAM to form correctly (M,N). (O–R) Results of the reciprocal experiment in which Gsc expression was analysed in E10.5 (O,P) and E15.5 (Q,R) Bapx1−/− embryos. Expression of Gsc is unaffected by the loss of Bapx1 expression within the first branchial arch. At E15.5 expression of Gsc is seen around Meckel’s cartilage (Me) and in the pharyngeal cleft between the first and second branchial arches (arrowhead).
**Bapx1 regulates patterning in the middle ear**

Along with many other defects in the developing middle ear, a fusion of the malleus and incus is seen in the *Eya1* mutants (Xu et al., 1999). *Eya1* expression has been previously described in the ear, but with little specific reference to the middle ear ossicles (Kalatzis et al., 1998). *Eya1* is not found in between the malleus and incus (i.e. in the joint region) as seen with *Bapx1*, but is expressed around the malleus and incus (Fig. 8D-F). Such an expression pattern supports the theory that the malleus and incus are originally derived from a single precartilaginous condensation (Goodrich, 1986), in a similar manner to that seen for the quadrate and articular in zebrafish and chick (Schilling and Kimmel, 1997; Wilson and Tucker, 2004). In the *Bapx1* mutant, the expression of *Eya1* was unaffected in the middle ear, agreeing with the fact that no fusion defect was seen (data not shown).

In zebrafish, the reduction of *Bapx1* expression results in loss of *Gdf5* expression in the developing jaw joint. *Gdf5* expression is downregulated in the cartilages of the jaw but remains high in the jaw joint itself. (Fig. 8D-F). By E9, expression of *Bapx1* is downregulated in the cartilages of the jaw but remains high in the jaw joint itself.

**Fig. 6.** Comparison of membranous bone ossification in the middle ear and jaw joint. Alcian Blue and Alizarin Red stained skeletal preparations. (A-C) Development of the cartilages and bones of the murine middle ear, side view. (D-F) Development of the cartilages and bones of the chick jaw joint, dorsal view. (A) Formation of the malleus and incus at E14. The malleus develops at the proximal end of Meckel’s cartilage. There is no bone ossification at this stage. (B) Ossification of the tympanic ring at the base of the malleus at E16. (C) Ossification of the gonium in between the malleus and tympanic ring at E18.5. (D) Formation of the jaw joint between the articular and the quadrate at E7. The articular lies at the proximal end of Meckel’s cartilage. (E) Ossification of the angular under the articular and Meckel’s at E9. (F) Ossification of the prearticular next to the angular at E13. I, incus; M, malleus; Me, Meckel’s cartilage; Ty, tympanic ring; G, gonium; A, articular; Q, quadrate; An, angular; P, prearticular.

**Fig. 7.** Expression of *Bapx1* in the chick jaw joint. (A) Section stained with Haematoxylin/Eosin. (C,E,H) Sections stained with Alcian Blue and chlorantine Fast Red. (B,D,F) 35S radioactive in situ hybridisation for *Bapx1*. (G) 35S radioactive in situ hybridisation for *Runx2*. (A,B,E-I) Frontal sections. (C,D) Sagittal sections. (A,B) E7. (C-G) E9. (H,I) E12. (A,B) Expression of *Bapx1* in the quadrate and articular cartilages of the jaw and in between the developing jaw joint (arrow). (C,D) By E9, expression of *Bapx1* is downregulated in the cartilages of the jaw but remains high in the jaw joint itself (arrow). (E) Frontal view showing the developing membranous bones associated with the articular. The presumptive prearticular is marked by an asterisk. (F) Expression of *Bapx1* coinciding with the region of the presumptive prearticular and around the angular and surangular bones surrounding the jaw joint. (G) Expression of the bone marker *Runx2* within the developing membranous bones (angular, surangular and prearticular). (H,I) Expression of *Bapx1* around the membranous bones surrounding the articular at E12 but not within these bones. A, articular; Q, quadrate; An, angular; S, surangular, P, prearticular.
encodes a TGFβ-related signalling factor expressed in early cartilage condensations and later in developing joints (Storm et al., 1994). A subset of mouse appendicular and axial joints requires GDF5 function (Storm and Kingsley, 1996). Therefore Gdf5 expression was examined in the developing joint region in between the malleus and incus in the mouse. At E15.5, when these two cartilages are clearly separated, Gdf5 expression was observed between the incus and malleus, overlapping the expression of Bapx1 (Fig. 8G,I). Gdf5 expression was therefore examined in the Bapx1 mutant mice. Surprisingly, given the zebrafish result, no effect on Gdf5 expression could be seen (Fig. 8J). Gdf6 was recently shown to be expressed between the incus and malleus during middle ear development and Gdf6 mutant mice have defects in the middle ear articulations not previously seen for Gdf5 mutants (Settle et al., 2003). Analysis of the expression of these two Gdf genes in the chick showed that both Gdf5 and Gdf6/7 (a single gene with homology to both murine Gdf6 and Gdf7) were expressed in the joint region between the quadrate and articular (Wilson and Tucker, 2004) (data not shown). In the mouse, Gdf6 expression pattern was similar to Gdf5 in the developing middle ear (Fig. 8H). No change in Gdf6 expression, however, was observed in the Bapx1 mutant mice (Fig. 8K). Therefore, the joint signalling cascade controlled by Bapx1, as identified in zebrafish, does not appear to be active in the mammalian middle ear. Continued expression of joint markers in between the malleus and incus can therefore explain the lack of a defect in this region in the mutant mice.

Discussion

**Bapx1 is required for middle ear development**

Bapx1 is an essential gene in the molecular control of middle ear development and detailed examination of the phenotype of Bapx1-null mice reveals potential roles for the gene in regulating both ossicle specification and morphogenesis. Bapx1 is expressed in the early condensations that correspond to the developing anterior part of the tympanic ring, gonium and malleus. In the absence of Bapx1, the gonium does not form and the tympanic ring is defective, with a portion of the anterior extremity of the ring absent, suggesting a role for Bapx1 in the specification of these elements. The malleus develops in Bapx1−/− mice, but does so abnormally, with the body of the ossicle appreciably narrower than in wild-type embryos. Bapx1 is therefore not involved in the initial specification of the malleus but rather may play a role later in the morphogenesis of this ossicle. However, the malleal phenotype in Bapx1−/− mice might also be interpreted as a result of a defect in cell specification, specifically in the population of cells located in the outer region of the mesenchyme surrounding this ossicle. The gonial/tympanic ring and the malleal defects in Bapx1−/− mice, which are...
manifest as defects in specification and morphogenesis, respectively, may therefore simply reflect timing differences; \textit{Bapx1} acting early in the specification of the gonium and anterior tympanic ring development and later in malleal development.

Our findings raise the possibility of a developmental pathway regulated by \textit{Bapx1} that is specific for defining the width of the malleus. The idea of several different developmental mechanisms that are each crucial for regulating distinct aspects of the development of the middle ear ossicles has been proposed (Mallo, 1997). Administration of RA to embryos at different stages of gestation suggests a temporal order to the patterning of the malleus (Mallo, 1997), although genetic studies, including those involving targeting of \textit{Gsc}, \textit{Msx1} and \textit{Prx1}, suggest the existence of discrete regulatory pathways for domains within the ossicle. The functional significance of malleal-width-specific pathway in the mouse is not immediately apparent; however, it is tempting to speculate that changes in the structure of the middle ear ossicles may have a bearing on aspects of an organism's hearing capabilities, as has been demonstrated with respect to tympanic-membrane area and middle ear structure (Rosowski, 1992).

**Link between presence of a gonium and frequency of hearing**

Postnatally, the gonium fuses with both the tympanic ring and the malleus and is referred to as the process folii (anterior or gracilis), a component of the malleus. Clearly, however, the gonium is an independently specified component of the middle ear. Studies into the auditory role of the gonium are scarce; however, consensus suggests that the gonium acts as a rigid link connecting the malleus to the tympanic bone (Fleischer, 1978). Analysis of audiograms taken from a variety of species suggest a relationship between the degree of fusion between the malleus and the tympanic bone, as mediated by the gonium, and the range of frequencies that the animal can detect (Fleischer, 1978; Rosowski, 1992). Animals with a complete gonium fusion hear at the higher end of the frequency spectrum. Those in which the gonium is underdeveloped or lacking entirely (e.g. human and rabbit), have a correspondingly weak link between the malleus and tympanic bone and consequently, are suggested to have an enhanced ability to hear lower frequencies of sound. It is tempting to speculate therefore, that although not rendering the mice deaf, the gonium hypoplasia observed in \textit{Bapx1} heterozygotes may have an effect on the range of frequencies that these mice can detect. Furthermore, we suggest that expression levels of \textit{Bapx1} may be important in different mammalian species in defining the auditory frequency range of detection.

**\textit{Bapx1} and \textit{Gsc} operate in combination in the gonium and tympanic ring**

In recent years, expression patterns for a number of genes in the first branchial arch have been described that appear highly regionalised and overlapping, raising speculation that the specific combination of genes expressed within any given region ultimately determines the developmental fate. The expression of \textit{Bapx1} and \textit{Gsc} is an example; these genes are expressed from E10.5 in an overlapping pattern, \textit{Bapx1} being more proximal than \textit{Gsc}. Inactivation of either \textit{Bapx1} or \textit{Gsc} results in defects associated with the tympanic ring. Although loss of \textit{Gsc} results in aplasia of this skeletal element, only the anterior-most thickening fails to develop in \textit{Bapx1}\textsuperscript{–/–} mice. The expression domains of both genes correlate well with the phenotypes observed in the knockout mice. Therefore, the cells destined to contribute to the tympanic ring appear to be identified early in embryogenesis by the expression domains of these two genes. \textit{Bapx1} and \textit{Gsc} act in concert to specify the thickened anterior end and thus are required for full structural composition of the tympanic ring. Our observations show clearly that in the branchial arch, \textit{Bapx1} and \textit{Gsc} are regulated independently in the region of overlap as deficiencies of either gene do not affect the expression of the other. In addition, \textit{Bapx1} does not regulate the expression of several other genes known to be involved in related aspects of middle ear development, including \textit{Prx1}, \textit{Msx1} and \textit{Hand2}.

\textit{Gsc} has both an inductive and a cell autonomous role in the formation of the tympanic ring. Expression in the EAM, the epithelium adjacent to the tympanic mesenchyme, is required for condensation; whereas chondrogenesis is cell autonomous (Rivera-Pérez et al., 1999). \textit{Bapx1} in the gonium and tympanic ring, however, appears to be required from an early stage of specification, as no signs of condensation or chondrogenesis are apparent.

**Evolution of the mammalian middle ear**

The evolution of the middle ear in tetrapods is well documented in the fossil record. The role of \textit{Bapx1} in the fish jaw suggested a corresponding role in the middle ear of mammals. As anticipated, \textit{Bapx1} does have a role in the skeletal pattern of the middle ear; however, it does not directly correspond to that predicted by the zebrafish data. To attempt to understand the molecular differences that may have occurred in the transition to the middle ear, \textit{Bapx1} expression was analysed in craniofacial morphogenesis in the developing chick. The facial skeletal pattern in chick is an amenable experimental system related to the transitional reptilian species important in the jaw/middle ear transformation (Allin, 1975). Such analysis enabled the comparison of expression patterns in relation to gross evolutionary changes in morphology. \textit{Bapx1} expression in the first branchial arch of vertebrates from fish to mammals reflects a continual role for the gene in craniofacial evolution. Theory holds that evolution of structure is founded on changes in gene regulatory systems. Hence, \textit{Bapx1} is likely to hold a strategic position in a regulatory network important in evolutionary change. Information from three vertebrate classes enabled an examination of the hypothesis that changes in regulatory networks are important in the morphological transformations.

Comparing the expression patterns of \textit{Bapx1} and \textit{Gsc}, the spatial organisation in the early branchial arch was indistinguishable in chick and mouse. At the initial stage of expression, \textit{Gsc} and \textit{Bapx1} overlap in the distal region of the mandibular arch in both mouse and chick. By E15.5, \textit{Bapx1} is expressed with the malleus and incus, the exact pattern depending on the part of the ossicle observed. Strong expression is detected between the malleus and incus at the site of articulation. At a similar developmental stage in chick (E7.5), \textit{Bapx1} is expressed in a comparable pattern, in between the quadrate and articular. Thus, even though the skeletal elements are distinct between species, similar characteristic \textit{Bapx1} and \textit{Gsc} spatial patterns are established in chick and...
mouse. In the midst of gross morphogenetic and functional changes, there is a continuity of spatial expression. Hence, the basis for patterning of the mandibular arch is unmodified in the transition to the middle ear.

Gdf5 and Gdf6 are also expressed in the region surrounding the incus and malleus in mouse, and articular and quadrate in chick. This pattern overlaps with the expression of Bapx1 in both species. In fish, Gdf5 expression is dependent on Bapx1, whereas in mouse, the Gdf expression is unaffected in the Bapx1<sup>−/−</sup> mutant. Thus, several changes of the Gdf family members have occurred. First, a second GDF family member, Gdf6, is expressed in the mouse in a comparable pattern of Gdf5; and second, the expression of neither Gdf gene is controlled by Bapx1. Although Bapx1 has an overall role in the size of the incus, the formation of the joint with the incus is independent of Bapx1 expression.

We suggest that Bapx1 spatial expression in branchial arch derivatives is continuous in vertebrate evolution; however, pertinent to the morphological transition are developmental processes downstream of the initial patterning events. Regulatory changes have occurred but these are to target genes of the regulatory network. Lack of control of Gdf expression by Bapx1 in mouse may reflect a number of downstream alterations that have occurred in the transition to middle ear ossicles and is a likely explanation for the differences in joint formation between mouse and fish. We suggest that the regulatory network involving the spatial expression of Bapx1 in vertebrate species is not detectably altered, but that the crucial evolutionary changes are downstream of the transcription factor and these are central to realisation of the species specific phenotypes.

We thank Chuck Kimmel for the informative discussions that lead to this work and for sharing unpublished data. We thank E. Vuorio, Pin-xian Xu and Jill Helms for the α1 (II) collagen, Eyal1 and Runx2 in situ probes, respectively. Thanks to James Sharpe and Carlo DeAngelis for assistance with OPT, Jamile Hazan for her help in cloning the chick Bapx1 probe used in this paper, and to David Wilkinson for providing the cDNA library.

References


