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Hoxa-2 restricts the chondrogenic domain and inhibits bone formation during development of the branchial area

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SUMMARY

In *Hoxa-2*$^{-/-}$ embryos, the normal skeletal elements of the second branchial arch are replaced by a duplicated set of first arch elements. We show here that *Hoxa-2* directs proper skeletal formation in the second arch by preventing chondrogenesis and intramembranous ossification. In normal embryos, *Hoxa-2* is expressed throughout the second arch mesenchyme, but is excluded from the chondrogenic condensations. In the absence of *Hoxa-2*, chondrogenesis is activated ectopically within the rostral *Hoxa-2* expression domain to form the mutant set of cartilages. In *Hoxa-2*$^{-/-}$ embryos the *Sox9* expression domain is shifted into the normal *Hoxa-2* domain. Misexpression of *Sox9* in this area produces a phenotype resembling that of the *Hoxa-2* mutants. These results indicate that *Hoxa-2* acts at early stages of the chondrogenic pathway, upstream of *Sox9* induction. We also show that *Hoxa-2* inhibits dermal bone formation when misexpressed in its precursors. Furthermore, molecular analyses indicate that *Cbfa1* is upregulated in the second branchial arches of *Hoxa-2* mutant embryos suggesting that prevention of *Cbfa1* induction might mediate *Hoxa-2* inhibition of dermal bone formation during normal second arch development. The implications of these results on the patterning of the branchial area are discussed.

Key words: Branchial arches, *Hoxa-2*, *Sox9*, *Cbfa1*, Skeletogenesis, Mouse

INTRODUCTION

Cranial neural crest cells play a pivotal role in the development of the craniofacial area (Noden, 1988; Le Douarin et al., 1993). These cells delaminate from the dorsal part of the developing neural tube and migrate into the branchial arches and frontonasal mass where they contribute to a wide variety of tissues, including bone and cartilage (Noden, 1988; Le Douarin et al., 1993). Cell tracing studies in mouse and chick embryos have shown that neural crest cells originating from different rostrocaudal levels migrate into specific areas of the developing face (Lumsden et al., 1991; Serbedzija et al., 1992; Osumi-Yamashita et al., 1994). For instance, the first branchial arch is populated by neural crest migrating from the caudal midbrain and the first two rhombomeres, while rhombomere 4 neural crest cells populate the second branchial arch. After arrival of these neural crest cells in the branchial arches, a series of epithelial-mesenchymal interactions guide their further development into a variety of structures specific for the various regions (Carlson, 1994). The core and symphysial portions of Meckel's cartilage, as well as the dentary bone, derive from the distal part of the first branchial arch. The proximal part of the first arch (Table 1) gives rise to the squamous bone, the dorsal end of Meckel's cartilage, two of the middle ear ossicles (malleus and incus) and the tympanic ring, which supports the tympanic membrane. From the second branchial arch, the third of the middle ear ossicles, the stapes, develops together with the styloid process and the lesser horn of the hyoid bone (Table 1).

Two alternative mechanisms underlie the development of craniofacial skeletal elements. Endochondral elements develop through the formation of cartilage templates, later replaced by bone tissue (endochondral ossification), whereas dermal (or intramembranous) elements result from the osteoblastic differentiation of cells located within the condensing mesenchyme which lay down osteoid tissue (intramembranous ossification). Skeletogenesis initiates with the induction of control genes that specify an osteogenic or chondrogenic fate in previously undifferentiated mesenchymal cells. These genes include *Sox9* for cartilage (Bell et al., 1997; Ng et al., 1997) and *Cbfa1* for dermal bone (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997). Epithelial-mesenchymal interactions are thought to play an important role in skeletal induction in the craniofacial area (Hall and Miyake, 1995), acting either by direct contact or through the release of diffusible factors. Subsequently, the morphogenesis of the areas fated to the

Table 1. Skeletal derivatives from the first and second branchial arches from wild-type and *Hoxa-2*$^{-/-}$ embryos

	Wild-type	*Hoxa-2*$^{-/-}$
First branchial arch	Malleus	Malleus
	Incus	Incus
	Tympanic ring	Tympanic ring
	Squamous bone	Squamous bone
Second branchial arch	Stapes	Malleus*
	Styloid process	Incus*
	Lesser horn hyoid	Tympanic ring*
		Squamous bone*
Otic capsule	Oval window	No oval window

*In mirror image arrangement.

skeletal pathways must be modulated to achieve the formation of functional structures. In recent years, several genes have been identified that play a role in the morphogenesis of the craniofacial skeleton. Some of them, such as *Endothelin-1*, *Msx1*, *gsc* and *Mhox*, may be involved in skeletal induction processes (Kurihara et al., 1994; Satokata and Maas, 1994; Rivera-Pérez et al., 1995; Yamada et al., 1995; Martin et al., 1995); others, including *Dlx1*, *Dlx2*, *Hoxa-2*, as well as *Mhox*, may play a role in the morphogenesis of the induced skeleton (Gendron-Maguire et al., 1993; Rijli et al., 1993; Martin et al., 1995; Qiu et al., 1995, 1997).

Gene inactivation experiments have shown that *Hoxa-2* plays a central role in the development of the skeletal elements of the second branchial arch (Gendron-Maguire et al., 1993; Rijli et al., 1993). In *Hoxa-2* null mutant embryos, the normal second branchial arch derivatives are replaced by a duplicated set of proximal first branchial arch elements (Table 1). Thus, *Hoxa-2* appears to divert the morphogenesis of the skeletal elements in the second arch from a first arch ground program. Any explanation of this process must account for basic differences in the morphogenetic plans of the first and second branchial arches. First, both endochondral and intramembranous ossification occurs in the first branchial arch, while only endochondral ossification takes place in the second branchial arch. Second, all the second arch derivatives arise from a single cartilaginous condensation, Reichert's cartilage, whereas proximal derivatives of the first branchial arch arise from several independent skeletogenic centers: endochondral condensations lead to the formation of the middle ear ossicles (malleus and incus), while the squamous bone and tympanic ring develop from their respective intramembranous condensations. Multiple centers also occur in the second branchial arch of *Hoxa-2* mutant embryos in which the tympanic ring (and probably also the squamous bone) develops from a condensation independently of the malleus and incus (Mallo and Gridley, 1996). Therefore, *Hoxa-2* normally reduces the condensation centers and permits only endochondral ossification to occur in the second arch.

So far it is not known how *Hoxa-2* directs the correct morphogenesis in the second branchial arch. Considering the involvement of other *Hox* genes in establishing segmental identity in a variety of systems (Krumlauf, 1994) and the fact that *Hoxa-2* is expressed in the neural crest migrating to the second but not to the first branchial arch (Krumlauf, 1993; Prince and Lumsden, 1994; Mallo, 1997), it has been proposed that *Hoxa-2* specifies, in the second arch neural crest, a developmental plan responsible for the formation of second arch-specific structures (Gendron-Maguire et al., 1993; Rijli et al., 1993). A prediction from this hypothesis is that the expression of *Hoxa-2* in the first arch should result in the development of second arch structures replacing those of the first arch (Rijli et al., 1993). However, previous results from our laboratory indicate that the retinoic acid-induced ectopic expression of *Hoxa-2* in the first branchial arch results in variable deletions of first arch proximal derivatives but no sign of change in segmental identity was evident (Mallo and Brändlin, 1997). This suggests that *Hoxa-2* acts as more than a positive selector gene in second arch morphogenesis.

We investigated the mechanisms by which *Hoxa-2* might influence the morphogenesis of second arch elements. We find that *Hoxa-2* is widely expressed in the second arch mesenchyme, but is excluded from the chondrogenic condensations. Furthermore, expression of *Sox9* in *Hoxa-2*$^{-/-}$ embryos is shifted ventrally into the normal *Hoxa-2* expression domain where it is normally not expressed. Using a transgenic approach, we show that this change in *Sox9* expression is indeed responsible for the phenotype of the cartilaginous elements in the second arch of the *Hoxa-2* mutants. In addition, *Hoxa-2* inhibits dermal bone formation in the second arch, and this inhibition is mediated by the prevention of *Cbfa1* expression in that area.

MATERIALS AND METHODS

Transgenic constructs

Hoxa-2::*SOX9* construct: a 0.8 kb *Bgl*II fragment of the *Hoxa-2* genomic region containing the enhancer driving *Hoxa-2* expression in the second branchial arch, in which the Krox-20 binding sites were inactivated (Nonchev et al., 1996), was cloned upstream of the minimal promoter from the adenovirus 2 late region (Conaway and Conaway, 1988). The human *SOX9* cDNA (Wagner et al., 1994) and the polyadenylation signal of SV40 were then introduced downstream of the minimal promoter.

Msx2::*Hoxa-2* construct: the murine *Hoxa-2* cDNA was cloned downstream of a 5.1 kb genomic fragment containing the *Msx2* enhancer/promoter (Liu et al., 1994). The SV40 polyadenylation signal was inserted downstream of the *Hoxa-2* cDNA.

The constructs were released from vector sequences, gel-purified using the Qiaquick gel extraction kit (Qiagen) and injected at a concentration of 2 ng/μl according to standard protocols (Hogan et al., 1994).

Embryonic analyses

For the identification and analysis of the transgenes, DNA was obtained from the viscerae of mouse fetuses according to the method of Laird et al. (1991). DNA (5 μg) was digested with *Bam*HI and the fragments were resolved in 0.8% agarose gels, blotted to ZetaProbe GT membranes (BioRad) and hybridized with the appropriate ^{32}P-labeled probes according to standard methods (Sambrook et al., 1989). Copy number was estimated by comparing the intensities of the transgenic and endogenous bands.

The genotyping of embryos and fetuses from *Hoxa-2*$^{+/-}$ intercrosses was performed by PCR as described by Gendron-Maguire et al. (1993).

Whole-mount in situ hybridization was performed according to the method of Wilkinson (1992), radioactive in situ hybridization as described by Smith and Gridley (1992), and non-radioactive in situ hybridization on paraffin sections as by Jostarndt et al. (1994) using digoxigenin-labelled probes. The following probes were used: *Cbfa1*:

a 0.6 kb *Hin*dIII/*Pst*I fragment of the cDNA (Ogawa et al., 1993), obtained by RT-PCR; mouse *Sox9:* the same as described by Wright et al. (1995), obtained by RT-PCR; human *SOX9*: a cDNA fragment containing the whole coding region (Wagner et al., 1994); *neo*: a 0.6 kb *Pst*I/*Xba*I fragment from pPGK*neo*bpA (Soriano et al., 1991); *Hoxa-2*: the same as described by Mallo (1997); *Mhox*: a 0.6 kb fragment containing the entire coding region (Cserjesi et al., 1992), obtained by RT-PCR.

Skeletal preparations were performed as described by Mallo and Brändlin (1997) on 18.5 days post coitum (d.p.c.) embryos.

RESULTS

As a step to understanding the role of *Hoxa-2* in the morphogenesis of the skeletal elements derived from the second branchial arch we analyzed its expression pattern, particularly in relation to the structures affected in *Hoxa-2*$^{-/-}$ mice. Although previous analyses indicated that *Hoxa-2* is expressed in the second branchial arch mesenchyme (Krumlauf, 1993; Prince and Lumsden, 1994; Mallo, 1997), those studies were performed at early developmental stages, and no detailed analysis of *Hoxa-2* expression during morphogenesis of the second branchial arch structures was conducted. Analysis at later developmental stages (10.5-14.5 d.p.c.) revealed that *Hoxa-2* is widely expressed throughout the second branchial arch mesenchyme (Fig. 1 and data not shown). However, the condensations forming the second arch skeletal elements, although at the earliest stages they might contain some *Hoxa-2*-expressing cells, were mostly negative for *Hoxa-2* expression (Fig. 1 and data not shown). For instance, the developing stapes can be initially identified in the proximal region of the second arch as a condensation surrounding the stapedial artery (Anson and Cauldwell, 1942); this condensation was negative for *Hoxa-2* expression (Fig. 1A,C,G,J). Conversely, *Sox9*, an early marker of chondrogenesis (Wright et al., 1995), was expressed in this condensation (Fig. 1E,H,K), confirming its cartilage-forming nature. Later, when the stapes interacts with the otic capsule to become inserted in the oval window, *Hoxa-2* is still expressed around it and extends its expression through the prospective wall of the otic capsule in the region of the oval window (Fig. 1I,J). The expression of *Sox9* in this area was again complementary to that of *Hoxa-2*: *Sox9* was detected in the rest of the otic capsule, but not in the oval window (Fig. 1K). The apparent complementarity of *Hoxa-2* and *Sox9* was also clear for other second arch structures. The developing styloid process contained *Sox9* transcripts, in agreement with its endochondral nature (Fig. 1N), but was negative for *Hoxa-2* expression (Fig. 1M).

The absence of detectable *Hoxa-2* expression in the skeletal

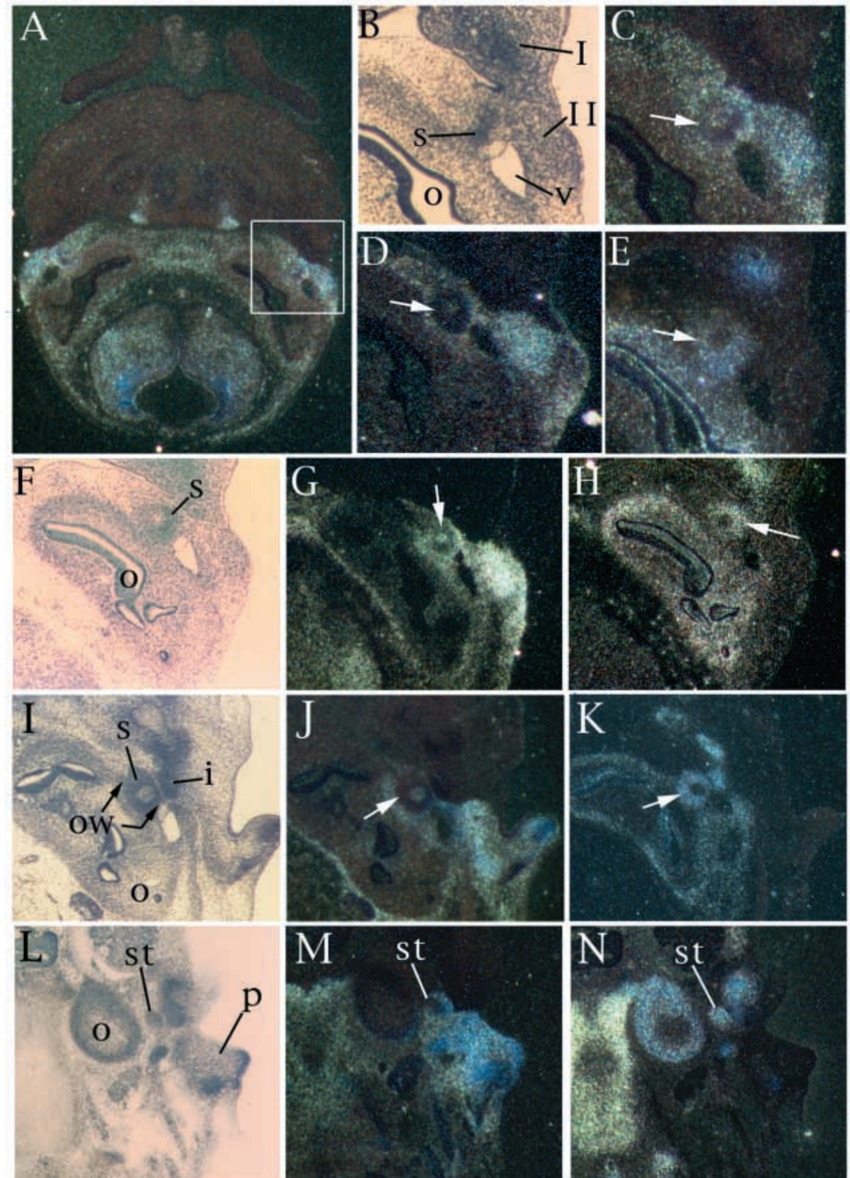

Fig. 1. Expression of *Hoxa-2*, *Sox9* and *neo* during the development of second arch structures. In situ hybridization was performed on transverse sections from 12.0 (A-E), 12.75 (F-H and L-N) or 13.5 (I-K) d.p.c. embryos with probes for *Hoxa-2* (A,C,G,J,M), *Sox9* (E,H,K,N) or *neo* (D). The *Sox9* probe was hybridized to sections adjacent to those used for *Hoxa-2* except in G and H, in which sections from similar regions of different embryos were used; *neo* was analyzed on a similar area from *Hoxa-2*$^{+/-}$ embryos. B,F,I and L are bright-field pictures of the fields shown in C, H, J and M. C shows a higher magnification of the boxed area in A. (A,C,G,J) *Hoxa-2* expression is excluded from the stapedial condensation (white arrows) in the second branchial arch (II). In the region of the otic capsule (o), *Hoxa-2* is expressed around the stapes (s) in the oval window (ow). (E,H,K) *Sox9* is expressed in all the developing cartilage of the area displaying a pattern complementary to that of *Hoxa-2*. (D) *neo* is also excluded from the stapedial condensation. (L,M,N) *Hoxa-2* expression is excluded from the developing styloid process (st) which is positive for *Sox9* expression. All micrographs are oriented rostral side up. i: incus; I: first branchial arch; s: stapedial condensation; v: primary head vein.

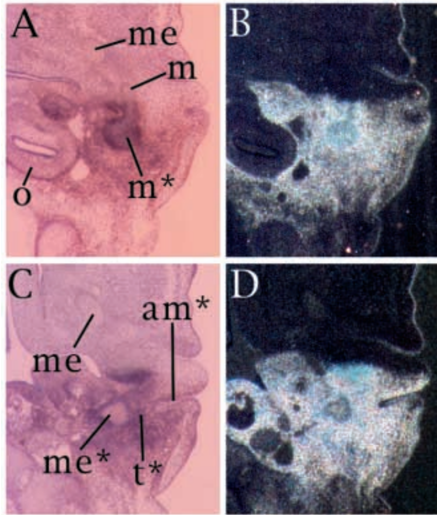

Fig. 2. Expression of *neo* in the second arch of *Hoxa-2*$^{-/-}$ embryos. In situ hybridization was performed on transverse sections of 13.5 d.p.c. *Hoxa-2*$^{-/-}$ embryos with a probe for *neo*. *neo* expression is seen in the region of the second branchial arch, including the condensations forming the duplicated malleus (m*), Meckel's cartilage (me*) and tympanic ring (t*). The otic capsule (o) and the normal malleus (m) and Meckel's cartilage (me) are negative. A and C show bright-field images of B and D, respectively. Micrographs are oriented rostral side up. am*, duplicated external acoustic meatus.

condensations makes it unlikely that this gene directs the morphogenesis of the second arch skeleton in a cell-autonomous fashion. Instead, the complementarity of *Hoxa-2* and *Sox9* expression domains (Fig. 1) and the finding that *Hoxa-2* is expressed in the oval window (Fig. 1J), a structure absent in *Hoxa-2*$^{-/-}$ animals (Gendron-Maguire et al., 1993; Rijli et al., 1993), suggest that *Hoxa-2* may inhibit skeletal formation in this area.

Based on this hypothesis, we speculated that the *Hoxa-2* mutant phenotype might derive from the development of skeletal elements within the *Hoxa-2* expression domain. To test this prediction, we took advantage of the finding that the expression of *neo* from the *Hoxa-2* null allele (Gendron-Maguire et al., 1993) resembled that of the native *Hoxa-2* gene (Fig. 1D; also reported by Rijli et al., 1993), indicating a dominant effect of the *Hoxa-2* second arch enhancer (Nonchev et al., 1996) on the PGK promoter. In *Hoxa-2* mutant embryos, *neo* expression was observed in the condensations of the second branchial arch that generate the duplicated set of first arch elements (Fig. 2). By contrast, the corresponding elements developing from the first arch, which are physically connected to those of the second arch (Gendron-Maguire et al., 1993; Rijli et al., 1993), were negative for *neo* expression (Fig. 2), emphasizing the specificity of the *neo* expression pattern. These results support the notion that the set of second arch elements in the *Hoxa-2* mutants arise from mesenchymal cells of the *Hoxa-2* expression domain.

Sox9 expression is altered in the second branchial arch of *Hoxa-2*$^{-/-}$ embryos

A variety of molecular and mutational studies have suggested the involvement of *Sox9* in the initial steps of cartilage development (Foster et al., 1994; Wagner et al., 1994; Wright et al., 1995; Bell et al., 1997; Ng et al., 1997). It is therefore possible that *Hoxa-2* exerts its negative effects on cartilage formation by modulating *Sox9* expression. In the second branchial arch, *Sox9* expression becomes detectable at 10.5 d.p.c. in the condensation of Reichert's cartilage (Wright et al., 1995; and Fig. 3A). Its expression domain is restricted to a central area in the branchial arch and shows in its dorsal part the typical perforated appearance of the stapedial condensation, which lies in close proximity to the otic vesicle (Fig. 3A,C,D). Analysis of *Sox9* expression in the second arch of *Hoxa-2* mutant embryos revealed a different pattern, already evident at 10.5 d.p.c. (Fig. 3E). Careful examination of sections and whole-mount preparations indicated that the *Sox9* expression domain is located more ventrally in the arch in *Hoxa-2*$^{-/-}$ embryos (Fig. 3; and data not shown) within the *Hoxa-2* expression region (compare Fig. 3G,H and Fig. 1A,C). The altered *Sox9* expression in *Hoxa-2*$^{-/-}$ embryos was particularly evident in the dorsal part of the *Sox9* expression domain, where it was no longer close to the otic vesicle but more ventrally located, adjacent to the first pharyngeal pouch (Fig. 3G,H), and its shape was different from that of the normal stapedial condensation (Fig. 3E). These results indicate that the

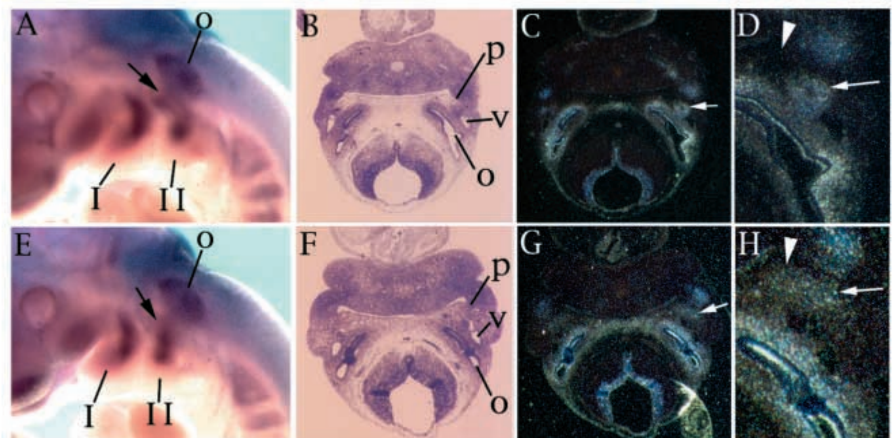

Fig. 3. Expression of *Sox9* in wild-type and *Hoxa-2*$^{-/-}$ embryos. *Sox9* expression was analyzed in wild-type and *Hoxa-2*$^{-/-}$ embryos in whole 10.5 dpc embryos (A,E) and in transverse sections from 11.5 d.p.c. embryos (C,D,G,H). B and F are bright-field pictures of the fields shown in C and G, respectively. D and H show higher magnifications of the ear region of sections C and G. (A,C,D) In the second branchial arch (II) of wild-type embryos, the dorsal part of the *Sox9* signal (arrow) shows a perforated appearance, in close association with the otic capsule (o). (E,G,H) In the *Hoxa-2* mutants, *Sox9* expression in the second branchial arch is altered. The dorsal part of its expression domain (arrow) shows a different appearance from that in the wild-type embryos and is located further away from the otic capsule, adjacent to the first pharyngeal pouch (p). The white arrowheads in D and H indicate the location of the endodermal epithelium associated to the second branchial arch in the first pharyngeal pouch. Micrographs are oriented rostral side up. I: first branchial arch; v: primary embryonic vein.

area where *Sox9* is induced in the second branchial arch is modulated by *Hoxa-2*.

Misexpression of *SOX9* in the *Hoxa-2* domain phenocopies *Hoxa-2*$^{-/-}$ fetuses

If the role of *Hoxa-2* in the morphogenesis of the cartilaginous components of the second arch is to determine the chondrogenic domain by restricting the area of *Sox9* induction, it is possible that forced expression of *Sox9* in the rostral *Hoxa-2* domain might generate a phenotype resembling that of the *Hoxa-2*$^{-/-}$ embryos. To test this possibility, we expressed *SOX9* under the control of the *Hoxa-2* second arch enhancer (Nonchev et al., 1996) in transgenic animals. We used a mutated version of this enhancer in which the Krox-20 binding sites are inactivated (Nonchev et al., 1996) to avoid complications from ectopic *SOX9* expression in the hindbrain.

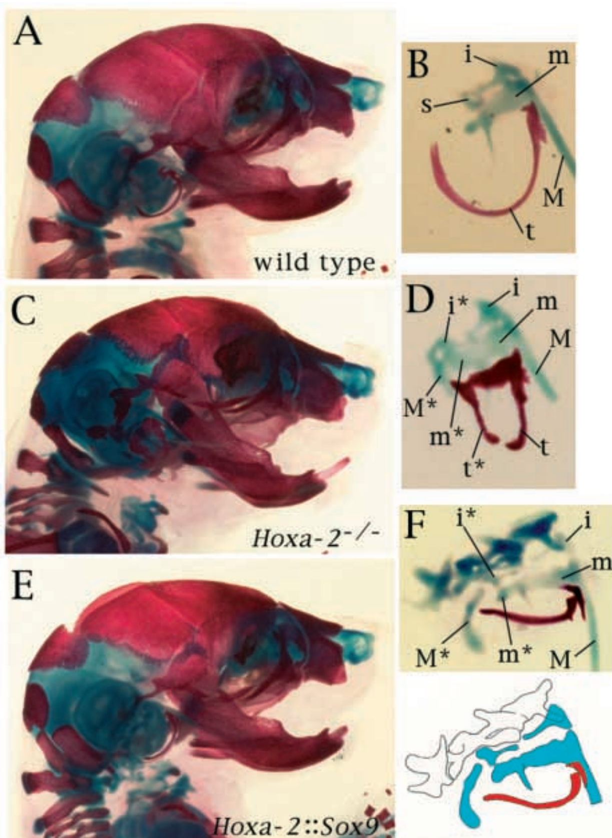

Fig. 4. Skeletal preparations of wild-type, *Hoxa-2*$^{-/-}$ and *Hoxa-2*::*SOX9* transgenic embryos. The skeletons of wild-type (A,B), *Hoxa-2*$^{-/-}$ (C,D) and *Hoxa-2*::*SOX9* transgenic (E,F) fetuses were stained with alcian blue and alizarin red at 18.5 d.p.c. A,C and E show the whole heads, and B,D and F, show the dissected ear elements. An explanatory diagram for F is included. (B) The ear region of wild-type embryos contain a malleus (m) still attached to Meckel's cartilage (M), the incus (i), stapes (s) and tympanic ring (t). (D) In the *Hoxa-2* mutant, the stapes is missing and the rest of the elements are duplicated (i, i*, m, m*, M, M*, t, t*). (F) In *Hoxa-2*::*SOX9* transgenic fetuses the region of the middle ear ossicles (in blue in the diagram) shows a structure resembling that found in *Hoxa-2* mutants. An extra cartilaginous mass is also present (in white in the diagram). The tympanic ring (red in the diagram) is not affected.

The effects of the transgene on the craniofacial region varied with the integration copy number. Fetuses containing low copy numbers (up to 10) of the transgene were indistinguishable from non-transgenic controls (not shown). However, phenotypic alterations were observed in fetuses containing higher copy numbers. Those alterations were restricted to the region derived from the second arch, with the rest of the craniofacial area showing a completely normal appearance (Fig. 4). The normal second branchial arch elements were absent and were substituted by a cartilaginous mass located between the otic capsule and the first arch skeletal elements on the rostral side, extending caudally into the neck region (Fig. 4E,F). The shape of these cartilaginous formations varied from one fetus to another without any obvious pattern. Interestingly, the phenotype of these transgenic fetuses also included extra elements associated with the first arch cartilages with patterns reminiscent of the ear region of *Hoxa-2* mutants (Fig. 4D,F). A cartilage resembling an incus in mirror image orientation with respect to its normal counterpart was present, associated with a distal extension of the malleus and with an extended cartilage located in a position similar to that of the duplicated Meckel's cartilage of the *Hoxa-2* mutant embryos (Fig. 4D,F). Therefore, it seems that expression of *Sox9* within the *Hoxa-2* domain is able to partially reproduce the *Hoxa-2*$^{-/-}$ ear phenotype. Analysis of *SOX9* expression in *Hoxa-2*::*SOX9* transgenic embryos indicated that its expression in the second arch of these embryos is not restricted to the rostral *Hoxa-2* domain, as *Sox9* is in the *Hoxa-2*$^{-/-}$ embryos, but encompasses the whole *Hoxa-2* expression domain in this arch (Fig. 5; compare with Fig. 3E). Therefore, although it cannot be formally ruled out that *SOX9* misexpression induced the *Hoxa-2*::*SOX9* transgenic phenotype via interference with general patterning mechanisms, it is very likely that the cartilages in the second arch of the *Hoxa-2*::*SOX9* fetuses resulted from a dominant chondrogenic activity of *SOX9* in different areas of this arch. In the most rostral areas, similar to the case in *Hoxa-2* null mutants, it would induce the observed *Hoxa-2*$^{-/-}$-like elements, while in more caudal and proximal regions of the second arch it would generate the extra mass of cartilage.

Together, the results presented so far indicate that the *Hoxa-2*-dependent alteration of *Sox9* expression in the second branchial arch plays an important role in the genesis of the *Hoxa-2* mutant phenotype. It can be concluded that *Hoxa-2* influences the morphogenesis of the second arch cartilages specifying the *Sox9* functional domain by an inhibitory mechanism.

Hoxa-2 inhibits bone formation

In addition to the respecification of the cartilaginous skeleton, the *Hoxa-2* mutant phenotype includes the duplication of first arch bony elements (Gendron-Maguire et al., 1993; Rijli et al., 1993). In *Hoxa-2*::*SOX9* transgenic fetuses, these dermal elements were not affected (Fig. 4). Thus, the duplicated tympanic ring and squamous bone of the *Hoxa-2*$^{-/-}$ embryos probably do not result from a change in *Sox9* expression. We previously showed that the duplication of the tympanic ring in the *Hoxa-2* mutants results from the induction of an additional ossification center in the region of the second branchial arch (Mallo and Gridley, 1996). Because *Hoxa-2* appears to inhibit cartilage formation, we asked whether it has similar effects on the formation of intramembranous bone. We first expressed

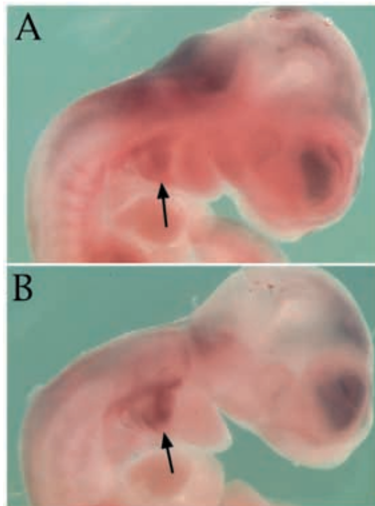

Fig. 5. Expression of *SOX9* in the second arch of *Hoxa-2:SOX9* transgenic embryos. (A) The expression of the human *SOX9* gene was analyzed on *Hoxa-2*:*SOX9* transgenic embryos at 10.5 d.p.c.; the transgene drives expression of *SOX9* to the *Hoxa-2* domain in the second branchial arch. Note the slight crosshybridization of the human *SOX9* probe with its murine homolog. (B) Control wild-type embryo showing the expression of *Hoxa-2*. Arrows indicate the second branchial arch.

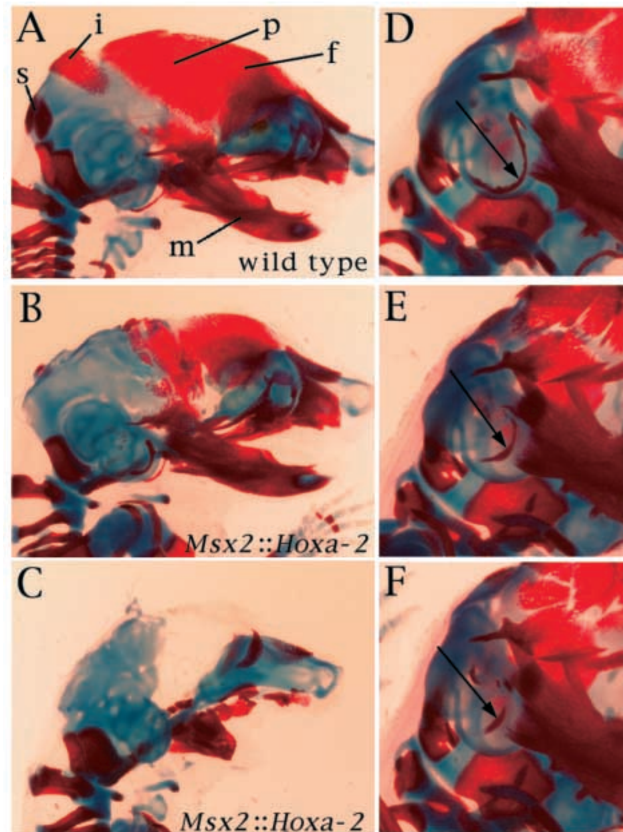

Fig. 6. Effect of *Hoxa-2* expression in dermal bones. Skeletons of wild-type (A,D) or *Msx2*::*Hoxa-2* transgenic embryos (B,C,E,F) were stained at 18.5 d.p.c. (B,C) The membranous bones of the skulls of the transgenic embryos were affected. The most commonly reduced or deleted were the interparietal (i), supraoccipital (s) and parietal (p) bones. (C) Very high copy numbers of the transgene induced nearly total deletion of the membranous bones of the craniofacial area. (D,E,F) The tympanic rings (arrow) were also typically reduced to various extents. f, frontal; m, mandible.

Hoxa-2 under the control of the *Msx2* gene promoter. This gene is expressed in the dermal bone precursors of the craniofacial area (MacKenzie et al., 1992) and a promoter fragment has been identified that drives expression in most of the *Msx2* expression domains (Liu et al., 1994). Skeletal preparations from *Msx2*::*Hoxa-2* transgenic embryos revealed a variable deficit of bone formation in the region of the skull (Fig. 6). This varied from small deficits in the interparietal bone and tympanic ring to extensive deletions of several bones in the craniofacial area. In an extreme case, most of the membranous bones in the craniofacial area were absent (Fig. 6C). Analysis of *Hoxa-2* expression in transgenic embryos at earlier stages of development showed that this gene was indeed ectopically expressed in the areas where the affected bones develop (Fig. 7), indicating a direct role of *Hoxa-2* in the transgenic phenotype. Furthermore, the extent to which bone formation was affected correlated with the transgene copy number, suggesting a direct relationship between the severity of the phenotype and *Hoxa-2* expression levels. These results indicate that *Hoxa-2* inhibits the formation of intramembranous bone in a dominant fashion. It should be noted that in addition to deficits in bone formation, chondrogenesis was also compromised in some of the *Msx2*::*Hoxa-2* transgenic fetuses, in particular those presenting strong dermal bone phenotypes (Fig. 6C). Given the negative effects of *Hoxa-2* on chondrogenesis, it is likely that the failure of some craniofacial cartilages to develop results from *Hoxa-2* expression in their primordia. Some support for this idea is provided by the cartilage phenotype in the fetus showed on Fig 6C: cartilage derived from the first branchial arch, where the *Msx2* enhancer is very active (Liu et al., 1994), are strongly affected, while the nasal capsule, which originates from a region of low *Msx2* enhancer activity, is essentially conserved.

To determine whether the inhibitory effects of *Hoxa-2* on dermal bone formation have physiological relevance, we analyzed *neo* expression during the development of the duplicated tympanic ring in the second arch of *Hoxa-2*$^{-/-}$ embryos. In fact, *neo* is expressed in the condensation that initiates this structure (Fig. 2D), indicating that the additional tympanic ring of *Hoxa-2*$^{-/-}$ embryos develops within the *Hoxa-2* expression domain. Thus, this gene might indeed inhibit the formation of this bone in wild-type animals.

Cbfa1 expression is upregulated in the second branchial arch of *Hoxa-2*$^{-/-}$ embryos

We next wanted to examine the mechanisms by which *Hoxa-2* inhibits the formation of dermal bone. We focused first on *gsc* and *Mhox* because they are required for the formation of the tympanic ring (Rivera-Pérez et al., 1995; Yamada et al., 1995; Martin et al., 1995) and thus, *Hoxa-2* could inhibit the formation of a duplicated tympanic ring by modulating any of these genes. However, we showed previously that *Hoxa-2* does not affect the expression of *gsc* (Mallo and Gridley, 1996), and the expression of *Mhox*, which includes domains in the first and second branchial arches, was also not affected by *Hoxa-2* (Fig. 8B,C).

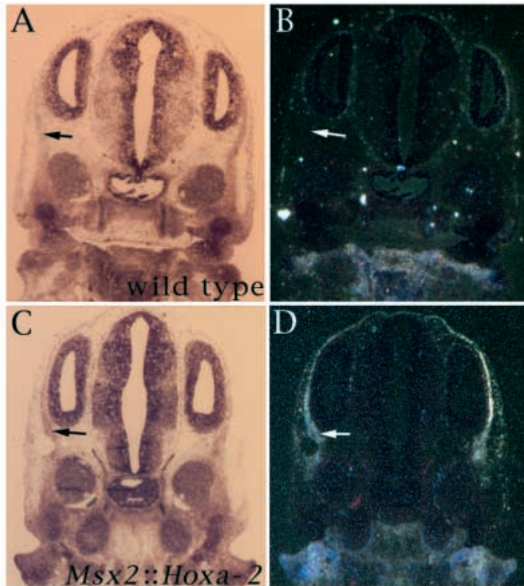

Fig. 7. Expression of *Hoxa-2* in the developing skull of *Msx2*::*Hoxa-2* embryos. *Hoxa-2* expression was analyzed on frontal sections of 13.5 d.p.c. wild-type (A,B) or *Msx2*::*Hoxa-2* transgenic embryos (C,D). Expression of *Hoxa-2* is detected in the mesenchyme forming the dermal bones of the skull (arrows) only in the transgenic embryos.

We then asked whether *Hoxa-2*, which had a clear effect on the expression of one of the earliest regulators of cartilage formation, *Sox9* (Graves, 1997), might also affect the expression of *Cbfa1*, a key regulator of membranous bone development (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997). At 11.5 d.p.c. *Cbfa1* is expressed in several osteogenic domains of the wild-type embryo, including the developing mandible and maxilla (Fig. 8D); in the ear region, expression was detected rostral to the pit where the external acoustic meatus originates (Fig. 8D,E). Analysis of sections at 12.5 d.p.c. (Fig. 8F) confirmed that the *Cbfa1*-expressing region corresponds to the site where the tympanic ring is initiated (Mallo and Gridley, 1996; Mallo, 1998). *Cbfa1* expression in the second arch of wild type embryos is very low, being detectable only upon over development of the whole-mount in situs (Fig. 8D,E; and not shown). In the *Hoxa-2* mutant, an additional expression domain with an intensity similar to that in the first arch, was observed caudal to the prospective external acoustic meatus (Fig. 8G,H) in an area where *Hoxa-2* is normally expressed (Fig. 8A). Analysis of sections at 12.5 d.p.c. (Fig. 8I) showed that this extra *Cbfa1* domain corresponds to the region where the supernumerary tympanic ring develops in the *Hoxa-2*$^{-/-}$ animals (Mallo and Gridley, 1996; Mallo, 1998). These results suggest that prevention of *Cbfa1* expression mediates the inhibition of dermal bone formation by *Hoxa-2*.

DISCUSSION

Hoxa-2 inhibits membranous bone and cartilage formation

Previous studies have shown that the *Hoxa-2* gene is essential for proper development of the skeletal structures derived from the second branchial arch (Gendron-Maguire et al., 1993; Rijli et al., 1993). When inactivated, the second branchial arch

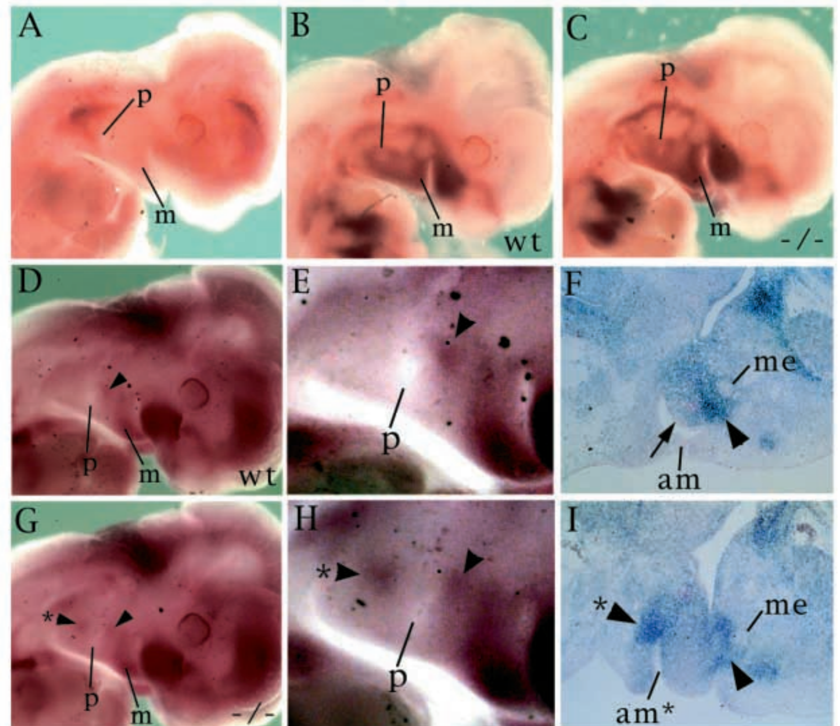

Fig. 8. Expression of *Hoxa-2*, *MHox* and *Cbfa1* in wild-type and *Hoxa-2*$^{-/-}$ embryos. In situ hybridization was performed on wild type (A,B,D,E,F) and *Hoxa-2* mutant (C,G,H,I) whole mount embryos at 11.5 d.p.c. (A-E,G,H) or on transverse sections of 12.5 d.p.c. embryos (F,I). The probes used detected *Hoxa-2* (A), *MHox* (B,C) and *Cbfa1* (D-I). E and H are higher magnifications of the branchial area of the embryos shown in D and G, respectively. (A) *Hoxa-2* is expressed in the second arch region, caudal to the pit of the prospective external acoustic meatus (p). (B,C) In the region surrounding the meatal pit (p), *MHox* expression is similar in wild-type (B) and *Hoxa-2* mutant (C) embryos. In both cases, it is expressed rostral and caudal to the pit. (D,E) In wild-type embryos, a domain of expression for *Cbfa1* (arrowhead) is detected rostral to the meatal pit (p). (G,H) In the *Hoxa-2* mutants additional *Cbfa1* expression is detected (arrowhead with asterisk) caudal to the meatal pit (p). (F) When analyzed in sections of wild-type embryos at 12.5 d.p.c. *Cfba1* is detected (arrowhead) in the first arch associated to the external acoustic meatus (am), in close proximity to Meckel's cartilage (me). Note that the medial surface of the acoustic meatus (arrow), which is starting to invaginate, is the caudal limit of first arch. (I) In *Hoxa-2* null mutants an additional *Cbfa1* expression domain is detected (arrowhead with asterisk) associated with the duplicated acoustic meatus (am*) in the second branchial arch. The micrographs in F and I are oriented rostral side to the right. m, developing mandible.

cartilages fail to develop and are replaced by a duplicate set of proximal first arch elements. Therefore, it has been suggested that, like other *Hox* genes in a variety of developmental settings (Krumlauf, 1994), *Hoxa-2* provides the second arch with regional identity (Gendron-Maguire et al., 1993; Rijli et al., 1993; Hall and Miyake, 1995). Our present results suggest that *Hoxa-2* does not provide the second arch skeletal elements with an intrinsic and specific developmental plan since, except for a few cells at the earliest stages, this gene is not expressed in the condensations that initiate and form those elements. Theoretically, it is still possible that *Hoxa-2* expression in those cells is sufficient to give a specific plan to the second arch condensations. However, our results rather suggest that *Hoxa-2* influences the morphogenesis of the second branchial arch elements by preventing dermal bone and cartilage formation in particular areas.

Cartilage is clearly induced in the second arch both in the presence and absence of *Hoxa-2*. However, our data indicate that the site of cartilage formation and the resulting set of structures differ depending on whether *Hoxa-2* is expressed. In wild-type embryos, chondrogenesis occurs in a group of *Hoxa-2*-negative cells in the core of the second branchial arch; this chondrogenic center generates the stapes and other second arch-derived cartilages. In *Hoxa-2*$^{-/-}$ embryos, chondrogenesis is activated in a different subset of second arch mesenchymal cells, located more ventrally within the typical *Hoxa-2* expression domain, resulting in a duplicated malleus and incus. Several findings support such an interpretation. *neo* (which is expressed from the mutant allele similar to *Hoxa-2*) is expressed in the chondrogenic condensations located in the second arch of *Hoxa-2*$^{-/-}$ embryos. In addition, in *Hoxa-2* null mutants the *Sox9* expression domain in the second arch, in particular its dorsal part, is shifted into the *Hoxa-2* 'territory'. Finally, when *SOX9* was expressed under the control of the *Hoxa-2* promoter in transgenic animals, the ear region of the resulting fetuses contained cartilaginous elements resembling those of *Hoxa-2* null mutants, indicating that upregulation of *SOX9* in the *Hoxa-2* domain can indeed mimic part of the *Hoxa-2* mutant phenotype. Therefore, *Hoxa-2* influences the formation of cartilaginous elements in the second branchial arch by restricting the chondrogenic areas.

In addition to cartilages (malleus and incus), the duplicated set of first arch skeletal elements in the second arch of *Hoxa-2*$^{-/-}$ embryos includes dermal bones (tympanic ring and squamous bone) (Gendron-Maguire et al., 1993; Rijli et al., 1993). Since no membranous ossification occurs in the second branchial arch of wild-type embryos, *Hoxa-2* must somehow interfere with the formation of dermal bone occurring in the second arch of *Hoxa-2* null mutants. This process is expected to involve mechanisms different from those affecting cartilage formation, because the tympanic rings and squamous bones were not affected in *Hoxa-2*::*SOX9* transgenic fetuses containing *Hoxa-2*$^{-/-}$-like cartilage elements in the second arch region. This finding also makes it unlikely that *Hoxa-2* normally respecifies bone condensations into the cartilage pathway. Instead, our experiments suggest that *Hoxa-2* inhibits dermal bone formation in this area. The ability of *Hoxa-2* to inhibit bone formation is evident from our transgenic experiments in which this gene was ectopically expressed in several osteogenic areas. Furthermore, the supernumerary tympanic ring arises in the second arch of *Hoxa-2*$^{-/-}$ embryos in the *neo* (i.e. *Hoxa-2*) expression domain, indicating that this mechanism operates normally in the second branchial arch. Moreover, *Cbfa1* is upregulated in the second branchial arch of *Hoxa-2*$^{-/-}$ embryos. Thus, the absence of membranous bone in the second arch of normal embryos is the result of inhibition in areas where it would be made in the absence of *Hoxa-2*.

The *Hoxa-2* mutant phenotype includes the deletion of the oval window, located in the otic capsule (Gendron-Maguire et al., 1993; Rijli et al., 1993). The absence of this structure is not simply the consequence of the failure of the stapes to develop, because oval windows can be found in the absence of this ossicle (Mallo, 1997). Our results provide a rationale for the formation of this structure. Because *Hoxa-2* is expressed in the area where the oval window is formed, it might render the cells in that area unable to respond to the signals that induce the cartilage surrounding the inner ear. The stapedial condensation would then interact with this area and become inserted in the window.

Our experiments indicate that *Hoxa-2* acts at very early stages of skeletogenesis since the expression of *Sox9* and *Cbfa1*, which are so far the earliest markers for the chondrogenic and osteogenic pathways (Graves, 1997; Ducy et al., 1997), is affected by *Hoxa-2* from the first moment these genes can be detected. However, we cannot be certain about the molecular mechanisms of the *Hoxa-2*-mediated inhibition of skeletal formation. One possibility is that *Hoxa-2* represses directly *Sox9* and *Cbfa1* transcription. Alternatively, or in addition, *Hoxa-2* could down regulate other genes that mediate the induction of *Sox9* and *Cbfa1* in the second branchial arch. Biochemical studies are necessary to distinguish between these possibilities.

Patterning of the branchial area

Mutational analyses have shown that *Hoxa-2* plays an important role in patterning the second branchial arch (Gendron-Maguire et al., 1993; Rijli et al., 1993), but exactly how this gene exerts its role has remained unclear. Grafting experiments in birds (Noden, 1983) have suggested that the cranial neural crest is largely prespecified before its migration. In this context it has been proposed that *Hoxa-2* provides the neural crest cells with a differentiation program to form the second arch elements (Gendron-Maguire et al., 1993; Rijli et al., 1993). According to this view, wild-type and *Hoxa-2*$^{-/-}$ embryos develop different skeletal structures in their second branchial arches because of the loss of this specific morphogenetic program in the mutants. However, our results do not support this view and suggest instead that *Hoxa-2* acts later by preventing chondrogenesis and dermal bone formation in specific areas.

We previously hypothesized that *Hoxa-2* modulates the response of the mesenchyme to skeletogenic signals provided locally by the branchial arches (Mallo and Brändlin, 1997). In the absence of *Hoxa-2*, the first and second branchial arches would be endowed with similar developmental potential in terms of their ability to respond to local patterning cues. In this 'basic state', equivalent areas of the first and second branchial arches would respond to one or several common skeleton-inducing signals located probably between both branchial arches. Dermal bone and cartilage developing from those induced areas would form elements with first arch characteristics and opposite polarity (Gendron-Maguire et al.,

1993; Rijli et al., 1993). The presence of *Hoxa-2* modifies the competence of the second arch mesenchyme to respond to inducing signals. Cells responding to those signals in the absence of *Hoxa-2* are rendered unresponsive by an inhibitory action of this gene. As a result, dermal bone formation is completely inhibited and the cartilage-forming area is spatially shifted into a *Hoxa-2*-deficient domain. As a consequence, typical second arch structures form.

This hypothesis is consistent with our data and with the *Hoxa-2* null mutant phenotype. The area of the second branchial arch in which chondrogenesis is induced is different in wild-type and *Hoxa-2* mutant embryos. In the mutant situation, cartilage is induced in a region within the *Hoxa-2* expression area. When this gene is present, cartilage is formed more dorsally in the second arch in a *Hoxa-2*-deficient region. Thus, *Hoxa-2* appears to restrict the domain of cartilage formation inhibiting the mesenchymal response to chondrogenic signals. Similarly, our results suggest that dermal bone is not formed in the second arch because *Hoxa-2* blocks the mesenchymal response to the corresponding signals.

This interpretation also provides an explanation for the mirror image disposition of the duplicated ear elements in the *Hoxa-2*$^{-/-}$ embryos. If these elements are the result of skeletal induction from a center located between the first two arches, signaling similarly to both arches, the induced elements are likely to develop with opposite polarity. The existence of such a signaling center, already suggested by Rijli et al. (1993), is supported by the expression of *gsc* in the first and second branchial arches (Gaunt et al., 1993), although it is only required in the first arch (Rivera-Pérez et al., 1995; Yamada et al., 1995).

An important step in further understanding the development of the branchial area will be the identification of the inducing factors. Considering that the cartilage-forming area in the *Hoxa-2* mutants is located closer to the signaling center than that in normal embryos, it is possible that, at least for the cartilage, diffusible molecules are involved in the induction which would be prevented from further diffusion upon activation of responsive cells. *Shh* and members of the *Wnt*, *BMP* and *FGF* gene families are prime candidates since they have already been identified as signaling molecules in a variety of developmental settings (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Laufer et al., 1994; Yang and Niswander, 1995; Vainio et al., 1993; Neubüser et al., 1997). Furthermore, several of these genes are expressed in the branchial area (Parr et al., 1993; Heikinheimo et al., 1994; Wall and Hogan, 1995; Helms et al., 1997; Neubüser et al., 1997), where they have been shown to participate in morphogenetic processes (Vainio et al., 1993; Helms et al., 1997; Neubüser et al., 1997). *Endothelin-1* is another candidate to play a role in the induction process: the inactivation of this gene, which encodes a secreted peptide (Yanagisawa et al., 1988) with a restricted expression between the branchial arches (Kurihara et al., 1994), results in craniofacial defects characterized by extensive deletions of the skeletal derivatives of the branchial arches, without obvious effects on the rest of the craniofacial area (Kurihara et al., 1994).

Extension of the model to other areas and evolutionary implications

A wide variety of homeobox-containing genes have been implicated in the development of skeletal elements in different body areas. It is possible that some of them function through mechanisms similar to those described here for *Hoxa-2*. For instance, null mutations in *MHox* and *Dlx2* produced alterations in the proximal region of the branchial arches (Martin et al., 1995; Qiu et al., 1995, 1997). In both cases, the phenotype includes the altered development of the incus, which remains attached to an extra cartilage in the lateral wall of the skull. It is thus possible that in particular areas, these genes also modulate the responsiveness of the mesenchyme to chondrogenic signals in achieving the proper morphogenesis of skeletal elements like the incus. In this respect, it should be noted that in the absence of *Dlx1* and *Dlx2*, *Sox9* was found to be upregulated in the molar-forming area of the maxilla, a region where *Dlx1* and *Dlx2* are normally expressed (Thomas et al., 1997). For other genes, however, the null phenotypes cannot be easily interpreted in terms of inhibitory mechanisms on chondrogenesis or osteogenesis. For instance, genetic data suggest that more positive mechanisms mediate the function of *Msx1* or *gsc* (Satokata and Maas, 1994; Rivera-Pérez et al., 1995; Yamada et al., 1995), and differential proliferation rates provide a simpler explanation for the role of *Hox* genes in the morphogenesis of the axial skeleton (Condie and Capecchi, 1994). Therefore, homeobox-containing genes in general, and *Hox* genes in particular, might act in different skeletogenic areas according to different mechanisms.

Finally, modulation of skeletogenic competence might represent a general mechanism for the evolution of the craniofacial area. It has been suggested that the *Hoxa-2* mutant phenotype includes atavistic elements (Rijli et al., 1993), and inactivation of *MHox* and *Dlx2*, which as discussed above might also down regulate skeletogenesis, produced phenotypes that have been interpreted as the region of the middle ear adopting a configuration reminiscent of the reptilian jaw joint (Martin et al., 1995; Qiu et al., 1995, 1997), the homolog of the middle ear in non-mammalian jawed vertebrates (Novacek, 1993). Therefore, these genes (and others) might be involved in the evolution of this region by modulating the areas competent to respond to skeletal inducing signals. The availability of mutant strains for those genes will facilitate the design of experiments to test these possibilities.

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