Development 130, 3403-3414

doi:10.1242/dev.00554

Mesenchymal patterning by *Hoxa2* requires blocking Fgf-dependent activation of *Ptx1*

Nicoletta Bobola[1], Marta Carapuço[2], Sabine Ohnemus[1], Benoît Kanzler[1], Andreas Leibbrandt[3], Annette Neubüser[3], Jacques Drouin[4] and Moisés Mallo[1,2,*]

[1]Department of Developmental Biology, Max-Planck Institute of Immunobiology, Freiburg, Germany
[2]Instituto Gulbenkian de Ciência, Oeiras, Portugal
[3]Research Institute of Molecular Pathology, Vienna, Austria
[4]Laboratoire de Génetique Moléculaire, Institut de Recherches Cliniques de Montréal, Montréal, Canada
*Author for correspondence (e-mail: mallo@igc.gulbenkian.pt)

Accepted 16 April 2003

SUMMARY

Hox genes are known key regulators of embryonic segmental identity, but little is known about the mechanisms of their action. To address this issue, we have analyzed how *Hoxa2* specifies segmental identity in the second branchial arch. Using a subtraction approach, we found that *Ptx1* was upregulated in the second arch mesenchyme of *Hoxa2* mutants. This upregulation has functional significance because, in *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos, the *Hoxa2*$^{-/-}$ phenotype is partially reversed. *Hoxa2* interferes with the *Ptx1* activating process, which is dependent on Fgf signals from the epithelium. Consistently, *Lhx6*, another target of Fgf8 signaling, is also upregulated in the *Hoxa2*$^{-/-}$ second arch mesenchyme. Our findings have important implications for the understanding of developmental processes in the branchial area and suggest a novel mechanism for mesenchymal patterning by Hox genes that acts to define the competence of mesenchymal cells to respond to skeletogenic signals.

Key words: *Hoxa2*, *Ptx1*, Hox genes, Branchial arches, Mesenchymal patterning, Fgf signaling

INTRODUCTION

Hox genes are essential for development of a wide variety of organisms throughout the whole phylogenetic tree (Carroll, 1995). One of the major functions of these genes is to provide segmental units of the embryo with a specific identity. This principle, first described in *Drosophila* (Lewis, 1978), applies to many other organisms, including vertebrates, and to a variety of body areas, such as the central nervous system, the axial skeleton, the branchial arches or the limbs (for reviews, see Krumlauf, 1994; Zakany and Duboule, 1999; Burke, 2000; Trainor and Krumlauf, 2001). Intensive work during the past decade has uncovered some important principles of Hox gene organization and function (Krumlauf, 1994; Zakany and Duboule, 1999; Burke, 2000; Trainor and Krumlauf, 2001). Organized in clusters, they are expressed in overlapping domains in a precise spatial and temporal sequence corresponding to the position of the gene within the cluster. Segmental identities are often determined by a particular combination of Hox genes in what has been called 'Hox codes'. The phenotypic alterations produced after perturbation of these codes by genetic or teratogenic means led to the identification of specific Hox genes that, alone or in combination, control particular developmental processes. In addition, genetic interactions between some of these genes have been revealed. However, little is known about how Hox gene activities are converted into morphogenetic processes. To understand these mechanisms, the genes functionally downstream of these transcription factors must be identified and analyzed. Identification of such downstream effectors is never an easy task, aggravated in the case of the Hox genes by their peculiar expression and functional characteristics.

Hoxa2 has unique features that make it a good model to address this issue. First, it the only Hox gene involved in segmental specification of the second branchial arch (Gendron-Maguire et al., 1993; Rijli et al., 1993; Barrow and Capecchi, 1999). Second, the area phenotypically affected is mostly well defined and coincides with one of the major expression domains of the gene (Prince and Lumsden, 1994; Nonchev et al., 1996; Mallo, 1997). Therefore, the analysis of the role of *Hoxa2* in second arch skeletogenesis is technically more feasible and the knowledge gained can provide insights into how other Hox genes control development in other body areas.

Hoxa2 is required for proper skeletal development in the craniofacial area (Gendron-Maguire et al., 1993; Rijli et al., 1993; Barrow and Capecchi, 1999). In vertebrates, this area develops in a quasi-segmental fashion. Development of the facial region can be considered to start with the production of cranial neural crest cells from the dorsal aspect of the developing brain (Le Douarin and Kalcheim, 1999). Crest cells originating at particular levels along the rostrocaudal axis migrate to populate specific areas of the frontonasal mass and

the branchial arches, the prospective face and neck (Serbedzija et al., 1992; Köntges and Lumsden, 1996). Increasing evidence indicates that signals from the pharyngeal endoderm provide patterning information to postmigratory crest cells (Couly et al., 2002) and that Hox genes negatively affect the ability of neural crest cells to interpret these signals to form skeletal elements (Kanzler et al., 1998; Couly et al., 2002). However, a primary role for neural crest cells in patterning processes has also been suggested on the basis of interspecies grafting experiments (Schneider and Helms, 2003). Whatever the precise mechanisms might be, it is clear that precise coordination of these processes results in the formation of specific structures from each of the prospective craniofacial areas. For example, neural crest cells from the caudal midbrain and the first two rhombomeres (r) populate the first branchial arch (Serbedzija et al., 1992; Köntges and Lumsden, 1996) to give rise to the mandible and part of the middle ear, in particular the malleus, incus and tympanic ring (Mallo, 1998). Likewise, cells migrating from r4 populate the second branchial arch to form the third middle ear ossicle, the stapes, along with the styloid process and the lesser horn of the hyoid bone (Mallo, 1998). *Hoxa2* exerts its function in the latter region, this also being the rostral limit of its expression in the developing face (Prince and Lumsden, 1994; Nonchev et al., 1996; Mallo, 1997).

In the absence of this gene, the second branchial arch develops abnormally, giving rise to skeletal structures resembling those normally developing from the first arch, but in a mirror image disposition with respect to their first arch orthologs (Gendron-Maguire et al., 1993; Rijli et al., 1993; Barrow and Capecchi, 1999). Previous work from our laboratory indicated that *Hoxa2* defines skeletal second arch identity by negatively restricting chondrogenic areas and blocking dermal ossification (Kanzler et al., 1998). By contrast, other investigators suggest an active role for *Hoxa2* as a selector gene able to initiate a second arch specific program (Grammatopoulos et al., 2000; Pasqualetti et al., 2000). It is then clear that, to be able to understand definitively which processes are under *Hoxa2* control and how this gene performs its job, it is essential to identify the downstream targets of the Hoxa2 transcription factor and to elucidate how they are regulated.

We have addressed this issue by using a subtraction approach and have identified *Ptx1* (*Pitx1* – Mouse Genome Informatics) as one of the mediators of *Hoxa2* functional activity. We further show that this gene is upregulated in the second branchial arch mesenchyme of *Hoxa2* mutants. Moreover, in $Hoxa2^{-/-}$;$Ptx1^{-/-}$ embryos, part of the *Hoxa2* mutant phenotype is reverted to wild type, demonstrating that upregulation of *Ptx1* is essential for the genesis of part of the *Hoxa2* mutant phenotype. As *Ptx1* expression is repressed by *Hoxa2*, the latter must interfere, directly or indirectly, with the *Ptx1* activation process. Our results show that this activation depends on Fgf signaling and suggest that *Hoxa2* interferes with this activity. We further find that *Lhx6*, another known Fgf8 target in the first branchial arch, is also upregulated in the second arch in the absence of *Hoxa2*, providing more evidence for a role for *Hoxa2* in the modulation of Fgf signaling. The implications of these findings toward understanding patterning processes in the branchial arches and Hox gene activity in general are discussed.

MATERIALS AND METHODS

Affimetrix chip profiling

Second branchial arches of E11.0 embryos from $Hoxa2^{+/-}$ intercrosses were dissected out and frozen immediately in a minimum volume of PBS. After genotyping the embryos, two pools were made of wild type and $Hoxa2^{-/-}$ branchial arches, and total RNA was extracted from each, using Trizol. cDNA was produced from these RNA preparations using reverse transcriptase, and labeled cRNA was synthesized by transcription in vitro. The labeled RNA was hybridized to Affimetrix U74A microarrays as recommended by the manufacturer.

Mutant and transgenic animals and embryos

The *Hoxa2* (Gendron-Maguire et al., 1993) and *Ptx1* (Lanctôt et al., 1999) mutant strains have been described previously. The *Fgf8;Foxg1-cre* mutant embryos were created by intercrosses of $Fgf8^{flox/flox}$ females and $Fgf8^{+/\Delta 2,3}$;$Foxg1^{cre/+}$ males (Meyers et al., 1998; Hebert and McConnell, 2000). *Msx2::Hoxa2* transgenics were generated by pronuclear injection as described (Kanzler et al., 1998).

In vitro culture of branchial arch explants

First and second branchial arches were dissected out from early E9.5 embryos and incubated on top of isopore filters soaked on DMEM without sodium bicarbonate, containing 20 mM HEPES, pH 7.2, 15% FCS, 50 units/ml penicillin and 50 μg/ml streptomycin. The epithelia were removed from the mesenchymes by controlled enzymatic treatment as previously described (Mallo et al., 2000). The Fgfr inhibitor SU5402 was applied at 7 mM or 13.5 mM (in DMSO) on AG 1-X2 beads (BioRad) previously soaked in the inhibitor solution for 2 hours. Fgf8 was applied at 1 mg/ml in heparin beads. When explants were made for $Hoxa2^{-/-}$ arches, embryos were obtained from $Hoxa2^{+/-}$ intercrosses, the branchial arches dissected out, and the genotype of each embryo tested on the yolk sac as previously described (Gendron-Maguire et al., 1993). One side of the embryo was incubated with the inhibitor and the other was used as a control. All explants were incubated for 24 hours at 37°C in an atmosphere of 5% CO_2/95% air. Then they were fixed in 4% PFA and processed for in situ hybridization.

Molecular and phenotypic analyses

Whole-mount in situ hybridization was performed as previously described (Kanzler et al., 1998), using *Hoxa2* (Mallo, 1997), *Ptx1* (Lanctôt et al., 1997), *Cbfa1* (Kanzler et al., 1998), *Fgf8* (Crossley and Martin, 1995), *Dlx2* (Bulfone et al., 1993) and *Lhx6* (Tucker et al., 1999). When *Msx2::Hoxa2* transgenic embryos were analyzed, E10.5 embryos (transgenics and controls) were cut in half and each half hybridized with a different probe (*Hoxa2* or *Ptx1*) to allow direct comparison. To section the specimens hybridized as whole mounts, the embryos were embedded in gelatin/albumin and sectioned with a vibratome at 30 μm. Skeletal phenotypes were analyzed by Alcian Blue/Alizarin Red staining as described previously (Mallo and Brändlin, 1997). Apoptosis was analyzed by TUNEL using the procedure described in Kanzler et al. (Kanzler et al., 2000). For histological analyses, embryos were fixed in Bouin's, dehydrated and embedded in paraffin. Sections (10 μm) were then stained with Hematoxylin and Eosin.

RESULTS

Ptx1 is upregulated in the second branchial arch of $Hoxa2^{-/-}$ embryos

To identify the molecular mediators of *Hoxa2* activity during development of the branchial area, we concentrated on the second branchial arch, as it is a major domain of *Hoxa2* expression and, accordingly, is the area where most of the

skeletal phenotypic traits of the null mutants appear (Gendron-Maguire et al., 1993; Rijli et al., 1993; Barrow and Capecchi, 1999). A comparison of mRNA contents between second arches of wild-type and *Hoxa2* mutant embryos using Affimetrix microarrays led to the identification of several differentially expressed clones. Among these, a few were directly confirmed as differentially expressed by in situ hybridization in wild-type versus *Hoxa2*$^{-/-}$ embryos (Fig. 1, and not shown) (see also Fig. 4B). Interestingly, all the differential clones confirmed so far showed an upregulation in mutant embryos. We initially concentrated on one of these genes, *Ptx1*, as it has been shown to be involved in first branchial arch development (Lanctôt et al., 1999; Szeto et al., 1999) and thus seems to be a good candidate to play a functional role in the second arch phenotype of *Hoxa2* mutant embryos.

In the branchial area of wild-type embryos, *Ptx1* expression is restricted to the first branchial arch; second arches do not express this gene (Fig. 1A-C) (Lanctôt et al., 1997). Strong first arch expression can be detected in the rostral epithelium at E9.0. However, *Ptx1* mRNA is not found in the underlying mesenchyme until later stages, being clear at E10.0 as a rostrocaudal stripe through the central part of the arch (Fig. 1B,C) (Lanctôt et al., 1997). In *Hoxa2* null mutant embryos *Ptx1* expression in the first branchial arch is similar to that in wild-type embryos, but an additional *Ptx1* expression domain can be clearly detected in the central part (perpendicular to the proximodistal axis) of the second arch starting at E10.5 (Fig. 1E). This expression is maintained at later developmental stages and resembles spatially the one observed in the first arch (Fig. 1E,F). However, in the second arch, *Ptx1* expression is restricted to the mesenchyme (Fig. 1E, inset). These results clearly indicate that *Hoxa2* is required for blocking *Ptx1* expression in the second arch mesenchyme, and that, in the absence of *Hoxa2*, this mesenchyme behaves similarly to that of the first arch, at least with regard to *Ptx1* expression.

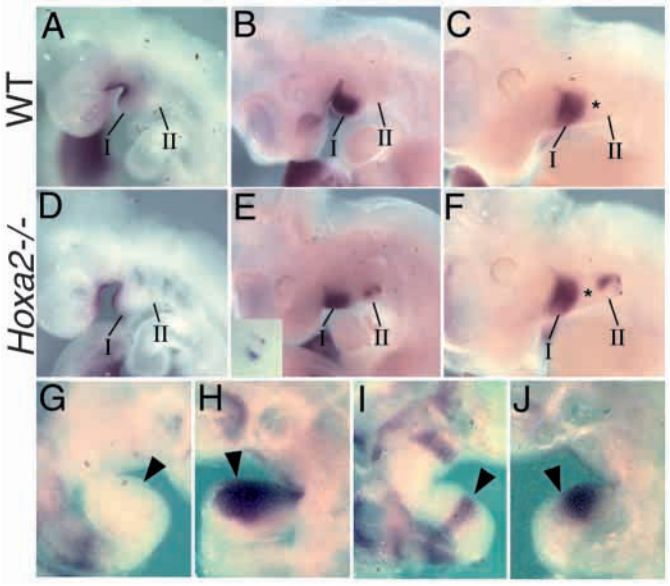

Fig. 1. *Ptx1* expression in the branchial area of wild-type, *Hoxa2*$^{-/-}$ and *Msx2::Hoxa2* transgenics. (A-F) *Ptx1* transcripts were detected in E9.5 (A,D), E10.5 (B,E) and E11.5 (C,F) wild-type (A-C) and *Hoxa2*$^{-/-}$ (D-F) embryos. In all cases, expression was detected in the first branchial arch (I). In the second arch (II) *Ptx1* was detected only in the *Hoxa2* mutant embryos after E10.5. (E, inset) Section through the second arch of a E10.5 *Hoxa2*$^{-/-}$ embryo showing that *Ptx1* expression was localized to the mesenchyme. The asterisks in C and F indicate the location of the external acoustic meatus. (G-J) E10.5 wild type (G,H) and *Msx2::Hoxa2* transgenics (I,J) were cut in halves. The right sides (G,I) were hybridized with a probe for *Hoxa2* and the left sides (H,J) with a probe for *Ptx1*. In the first arches (arrowheads) *Hoxa2* was expressed in the *Msx2::Hoxa2* transgenic (I) but not in wild-type (G) embryos. The *Ptx1* expression domain is larger in wild-type (H) than in *Msx2::Hoxa2* transgenic (J) embryos.

To determine whether *Hoxa2* could also act dominantly in the first arch mesenchyme to block *Ptx1* expression, we expressed *Hoxa2* in this mesenchyme in transgenic embryos, using the *Msx2* promoter (Kanzler et al., 1998). When compared with wild-type littermates, *Ptx1* expression was considerably reduced in the first arches of transgenic embryos in the areas corresponding to the ectopic domain of *Hoxa2* expression (Fig. 1G-J). This result shows that as in the second arch, *Hoxa2* is sufficient to downregulate mesenchymal *Ptx1* expression in the first arch, indicating that these two arches are equally competent to express or downregulate *Ptx1* in the absence or presence of *Hoxa2*, respectively.

Hoxa2 blocks Fgf8-dependent *Ptx1* induction

Hoxa2 blocks mesenchymal expression of *Ptx1* in the branchial arches, physiologically in the second arch but also in the first when ectopically expressed there. This implies that *Hoxa2* interferes directly or indirectly with some activating mechanism. In the branchial area, mesenchymal gene expression is often induced by interactions with the epithelia (Thesleff et al., 1995). If this is also the case for *Ptx1*, *Hoxa2* could be interfering with this activation mechanism.

To understand if mesenchymal *Ptx1* induction requires interactions with the epithelia, we dissected out branchial arches before *Ptx1* is expressed in the mesenchyme (E9.25 to E9.5) and incubated them in vitro with or without their epithelia. After 1 day, *Ptx1* was detected in the first branchial arches that had been incubated with ectoderm (n=8) (Fig. 2B) but not in the first arches whose ectoderms were removed before culture (n=6) (Fig. 2A). These results indicate that initiation of *Ptx1* expression in the branchial arch mesenchyme is dependent on epithelial signals. Understanding the nature of this inducing process could shed light into the mechanism of *Hoxa2* action. Interestingly, the spatial *Ptx1* expression in the intact explants resembled that observed in E10.5 wild-type embryos, being restricted to the central part of the first arch and with no detectable expression in the second arch. Hence, the control mechanisms for *Ptx1* expression seem to be largely conserved under our culture conditions, suggesting that the in vitro system could be used to address specific aspects of the induction process.

The *Ptx1* expression pattern in the first arch suggests that it might be dependent on Fgf signals. In support of this view, Fgf8-soaked beads were able to induce *Ptx1* mesenchymal expression in explanted first arches deprived of their epithelia (n=6 out of 8) (Fig. 2D) (St Amand et al., 2000). To determine whether Fgf8 is required for mesenchymal expression of *Ptx1* in vivo, we chose a genetic approach and analyzed *Ptx1* expression in *Fgf8;Foxg1-cre* mutant mice (A.L. and A.N., unpublished). In these mutant mice, which express cre from the

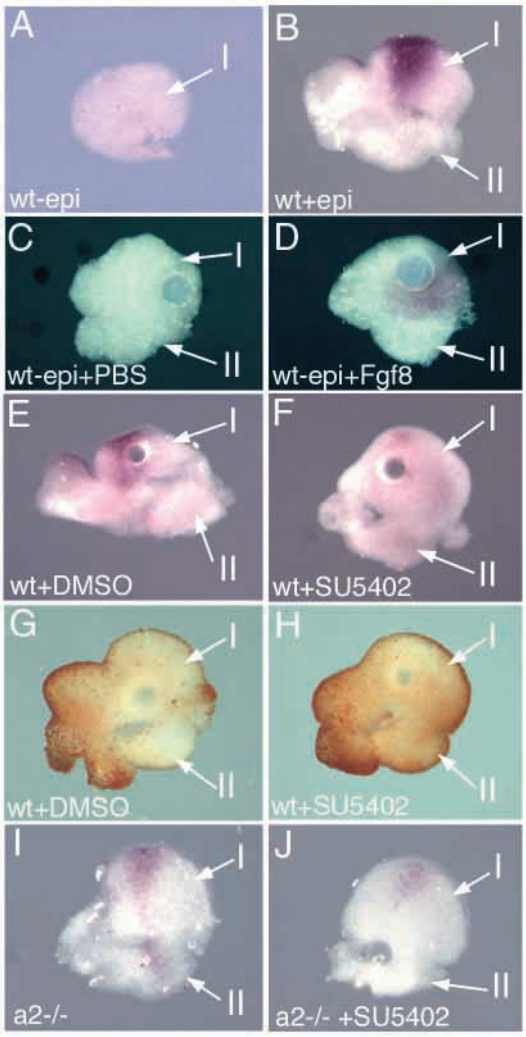

Fig. 2. Regulation of *Ptx1* expression by Fgf. Early E9.5 branchial arches were dissected out and incubated for 24 hours on isopore filters, and *Ptx1* expression was determined by in situ hybridization (A-F,I,J), or apoptosis was determined by TUNEL (G,H). (A) Wild-type first arch mesenchyme incubated without epithelium. (B) Wild-type first (I) and second (II) arches incubated with epithelium. (C) Wild-type first and second arches incubated without epithelium in the presence of a bead soaked in PBS. (D) Wild-type first and second arches incubated without epithelium in the presence of a bead soaked in 1 mg/ml Fgf8. (E) Wild-type first and second arches incubated with epithelium in the presence of a bead soaked in DMSO. (F) Wild-type first and second arches incubated with epithelium in the presence of a bead soaked in 13.5 mM SU5402. There is only residual *Ptx1* expression in the first arch. (G) TUNEL assay on wild-type first and second arches incubated with epithelium in the presence of a bead soaked in DMSO. (H) TUNEL assay on wild-type first and second arches incubated with epithelium in the presence of a bead soaked in 13.5 mM SU5402. (I) *Hoxa2*$^{-/-}$ first and second arches incubated with epithelium. (J) *Hoxa2*$^{-/-}$ first and second arches incubated with epithelium in the presence of a bead soaked in 13.5 mM SU5402.

Foxg1 locus (Hebert and McConnell, 2000), *Fgf8* is deleted from the branchial arch epithelia and the molecular and skeletal first branchial arch phenotype recapitulates that of the *Fgf8;Nes-cre* mutants (Trumpp et al., 1999). A previous study of the latter mutants showed that Fgf8 is not required for epithelial *Ptx1* expression (Trumpp et al., 1999). However, this study was restricted to E9.5 embryos and at this embryonic stage mesenchymal *Ptx1* expression cannot be properly evaluated. Therefore, we analyzed *Ptx1* expression at E10.5. Consistent with results of Trumpp et al. (Trumpp et al., 1999), *Ptx1* expression in *Fgf8;Foxg1-cre* mutants is not affected in the rostral epithelium of the first arch (Fig. 3B,C). However, mesenchymal *Ptx1* expression could not be detected in these embryos (Fig. 3B,C). The absence of *Ptx1* expression in the first arch mesenchyme is not simply due to the absence of cells that would express this gene, because other genes expressed in the same area [*Dlx2* (Fig. 3E); *Lhx7* (Trumpp et al., 1999)] can still be detected in the mesenchyme of E10.5 embryos that lack *Fgf8* expression in the first arch epithelium. These results show that Fgf8 is required for *Ptx1* expression in the first arch mesenchyme in vivo.

Considering the practical difficulties of generating *Fgf8;Foxg1-cre* mutants in a *Hoxa2*$^{-/-}$ background, and given that it is not clear whether Fgf8 would be removed from the relevant areas, we decided to analyze possible interactions between *Hoxa2* and Fgf signaling using the in vitro explant system. When E9.5 branchial arches were dissected out and incubated in vitro with a bead containing SU5402, an inhibitor of Fgf receptors (Mohammadi et al., 1997), *Ptx1* expression was strongly inhibited (n=8) (Fig. 2F) in a dose-dependent manner (not shown). This inhibition is specific, as control beads containing DMSO (the diluent) (n=4) had no effects on *Ptx1* expression (Fig. 2E). In addition, SU5402-mediated *Ptx1* downregulation seems not to be the consequence of increased apoptosis (Fig. 2G,H). In summary, the in vitro results with the Fgfr inhibitors reproduced the in vivo findings with the *Fgf8;Foxg1-cre* mutant mice.

When we explanted and incubated E9.5 *Hoxa2*$^{-/-}$ first and second branchial arches, we found that *Ptx1* was upregulated in both the first and second arches, thus reproducing the in vivo findings (n=3) (Fig. 2I). When these explants were incubated in the presence of SU5402, *Ptx1* expression was completely abolished from the second arch and reduced in the first (n=3) (Fig. 2J). These results indicate that inhibition of Fgf signaling in the second arch of *Hoxa2*$^{-/-}$ mutants reverts *Ptx1* expression to the wild-type (*Hoxa2*-expressing) situation. These findings, together with those obtained in the first branchial arch, assign *Ptx1* under the control of Fgf signaling and suggest that modulation of this signaling pathway could be the mechanism of *Ptx1* inhibition by *Hoxa2*.

Lhx6 and *Fgf8* expression in second branchial arches of *Hoxa2*$^{-/-}$ embryos

The above results clearly show that *Hoxa2* blocks *Ptx1* expression in the branchial arch mesenchyme. This could be via a direct effect on the *Ptx1* promoter (blocking activity of a transcriptional activator) or via an indirect effect, most likely by interfering with some step of the Fgf signaling pathway. Despite extensive studies on the *Ptx1* promoter, we have so far been unable to obtain any evidence for direct *Hoxa2* control. Therefore, the indirect hypothesis seems to be favored at the moment. If, indeed, *Hoxa2* controls *Ptx1* expression by modulation of Fgf signaling, the inhibitory effects of *Hoxa2* in the second arch should not be restricted to *Ptx1* alone but might also be extended to other Fgf targets. Previous analysis on

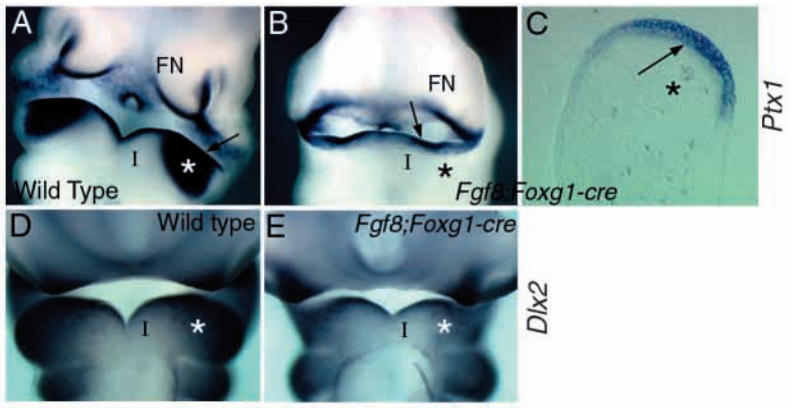

Fig. 3. Fgf8 is required for mesenchymal *Ptx1* expression. (A-C) *Ptx1* expression was analyzed by in situ hybridization at E10.5 in wild-type (A) and *Fgf8;Foxg1-cre* mutant (B,C) embryos. (A,B) A whole-mount staining; (C) a section through the first branchial arch of a stained embryo. Although the ectodermal expression in the rostral first arch epithelium (arrows A-C) was conserved in the mutant, no mesenchymal expression (asterisks, A-C) was observed. (D) *Dlx2* is expressed in the branchial arches and frontonasal mass of E10.5 wild-type embryos. Asterisk indicates the area where *Ptx1* is also expressed. (E) In E10.5 *Fgf8;Foxg1-cre* mutant embryos, *Dlx2* expression is still detected (asterisk), regardless of the smaller size of the first branchial arch (I). FN, frontonasal mass.

Fgf8;Nes-cre and *Fgf8;Foxg1-cre* mutant embryos revealed that *Lhx6* expression in the first arch mesenchyme requires Fgf8 (Trumpp et al., 1999) (A.L. and A.N., unpublished). Analysis of the gene chip data revealed that *Lhx6* was moderately upregulated (2.6-fold) in the second arches of *Hoxa2* mutants. Consistent with this finding, in situ analysis in *Hoxa2*$^{-/-}$ embryos revealed that *Lhx6* is indeed upregulated in the rostroproximal mesenchyme of the mutant second arches (Fig. 4A,B). Interestingly, this domain is located just beneath an area of *Fgf8* expression in the rostral second arch epithelium (Fig. 4C) and corresponds to one of the areas of strong *Hoxa2* expression in the second arch mesenchyme (Fig. 4E). These results clearly indicate that other genes under Fgf8 control are also upregulated in the absence of *Hoxa2* and further substantiate the role of *Hoxa2* in modulating Fgf signaling.

Hoxa2 repression of Fgf8 targets in the mesenchyme could occur by modulating the Fgf signaling pathway in the Fgf target cells (i.e. the mesenchyme) or by modulation of epithelial *Fgf8* expression. To test for the latter possibility, we compared *Fgf8* expression in wild-type and *Hoxa2* mutant embryos. As mentioned above, *Fgf8* expression can be detected in the second arch epithelium, in particular in areas corresponding to the caudal second arch ectoderm and in the proximal part of the first pharyngeal cleft (Fig. 4C). This expression pattern seemed unaffected by the presence or absence of *Hoxa2* (Fig. 4D). We cannot rule out the possibility that *Hoxa2* might affect expression of some other member of the large Fgf family. However, as Fgf8 seems to be the physiological activator of *Lhx6* and *Ptx1* (Trumpp et al., 1999) (Fig. 6), these results suggest that *Hoxa2* blocks Fgf signaling by interfering with the signaling pathway in mesenchymal (i.e. target) cells rather than affecting Fgf expression itself.

Looking at the *Fgf8* expression domains, a caveat must be raised regarding the relationship between Fgf8 signaling and *Ptx1* expression, as, contrary to *Lhx6*, no *Ptx1* transcripts can be detected in the anteroproximal mesenchyme of the *Hoxa2* mutant second arch. A possible explanation of this situation could be that *Ptx1* and *Lhx6* share a dependence on Fgf induction, but not on other control mechanisms. In the first arch, although *Lhx6* was detected beneath the whole *Fgf8*-expressing rostral ectoderm, *Ptx1* transcripts were detected only in the central area of *Fgf8* expression. Bmp4 may repress *Ptx1* expression in a dominant fashion, accounting for the absence of distal first arch expression (St Amand et al., 2000). A similar mechanism could be responsible for the absence of proximorostral *Ptx1* expression in the *Hoxa2* mutant second arch, as *Bmp4* is also expressed in the first cleft/pouch area (Fig. 4F) (Wang et al., 2001).

Partial rescue of the *Hoxa2*$^{-/-}$ phenotype in *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos

The above results indicate that in normal embryos *Hoxa2*

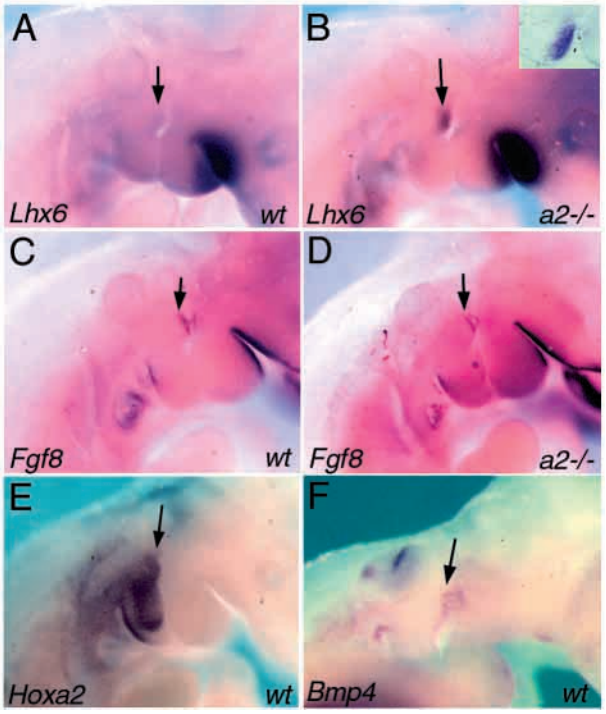

Fig. 4. *Lhx6*, *Fgf8*, *Hoxa2* and *Bmp4* expression in the branchial area. (A,B) Expression of *Lhx6* in E10.5 wild-type (A) and *Hoxa2*$^{-/-}$ (B) embryos. The arrow indicates the extra expression domain in the second arch mesenchyme beneath the rostral epithelium. The inset in B shows a section through the *Lhx6*-positive domain of the *Hoxa2* mutant second arch, to demonstrate that expression is in the mesenchyme. (C,D) Expression of *Fgf8* in E 10.5 wild-type (C) and *Hoxa2*$^{-/-}$ (D) embryos. The arrow indicates expression in the epithelium of the first cleft/pouch. (E) Expression of *Hoxa2* in E10.5 embryos. The arrow indicates the strong expression in the mesenchyme beneath the cleft/pouch epithelium. (F) Expression of *Bmp4* in E9.5 wild-type embryos. The arrow indicates the expression in the cleft/pouch.

prevents *Ptx1* expression in the second arch mesenchyme. As the absence of *Hoxa2* leads to a skeletal phenotype in the second arch (Gendron-Maguire et al., 1993; Rijli et al., 1993; Barrow and Capecchi, 1999) and *Ptx1* is involved in skeletal development (Lanctôt et al., 1999; Szeto et al., 1999), it is possible that the *Hoxa2* mutant phenotype is associated, totally or partially, with the *Ptx1* upregulation. To test if this is indeed the case, we generated *Hoxa2*$^{-/-}$ mice in which *Ptx1* upregulation could not occur (*Hoxa2*;*Ptx1* double mutants). Skeletal analysis of newborn double mutants revealed the presence of a single tympanic ring (Fig. 5D,F) instead of the two observed in *Hoxa2*$^{-/-}$ mice (Fig. 5B). The gonial bone (a part of the malleus that develops by dermal ossification), which is abnormally extended in *Hoxa2*$^{-/-}$ embryos (Fig. 5B), seemed to be connected in *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos to the extra dermal element associated with Meckel's cartilage observed in both the *Ptx1*$^{-/-}$ and *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos (Fig. 5C,D). The rest of the skeletal phenotype of these double mutants was an additive mix of *Hoxa2*$^{-/-}$ and *Ptx1*$^{-/-}$ characteristics (Figs 5, 6). For example, similar to *Hoxa2* single mutants (Gendron-Maguire et al., 1993; Rijli et al., 1993; Barrow and Capecchi, 1999), the incus, malleus and squamous bone were clearly duplicated in the double mutant (Fig. 5; Fig. 6K,L), and the basisphenoid presented the typical laterodorsal extension that connects with the duplicated incus (Fig. 6C,D). Other typical *Ptx1* mutant characteristics, such as the reduced mandible or the proximal extra dermal element associated with Meckel's cartilage (Lanctôt et al., 1999), also seemed unaffected by the *Hoxa2* mutation (Fig. 5C,D; Fig. 6F,H), and the hindlimb phenotype of *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos resembled that of their *Ptx1*$^{-/-}$ littermates (Lanctôt et al., 1999; Szeto et al., 1999).

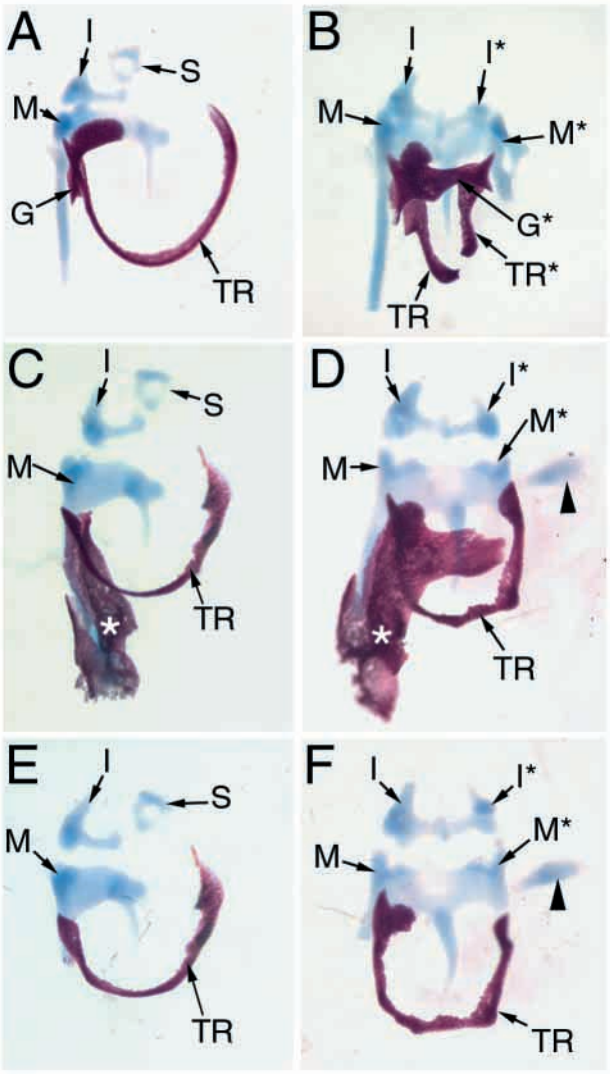

Fig. 5. Middle ear skeletal phenotype of *Hoxa2*$^{-/-}$, *Ptx1*$^{-/-}$ and *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ mutants. Stained middle ear skeletal elements from wild-type (A), *Hoxa2*$^{-/-}$ (B), *Ptx*$^{-/-}$ (C,E) and *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ (D,F) were dissected out. (B) In *Hoxa2*$^{-/-}$ embryos the malleus (M), incus (I) and tympanic ring (TR) were duplicated in mirror image disposition (M*, I* and TR*). The gonial bone was abnormally extended (G*). (C) In *Ptx1*$^{-/-}$ embryos, the tympanic ring was slightly deformed and there was an extra ossified mass (asterisk). (D) In *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ only the tympanic ring was not duplicated. The arrowhead points to a small chondrogenic mass associated to the duplicated malleus. (E,F) The same structures as in C and D but without the extra ossified mass. At least four embryos corresponding to each genotype have been analyzed and showed similar phenotypes.

The above skeletal analyses reveal that in the absence of *Ptx1*, the tympanic ring is not duplicated anymore in *Hoxa2*$^{-/-}$ embryos. To understand if this represented a true rescue of the *Hoxa2* mutant phenotype, we analyzed the formation of this structure in the mutants at earlier developmental times. At E16.6, *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos showed two tympanic ring primordia (Fig. 7C), indicating that, although they eventually fuse to form a single ring, they are formed from two different ossification areas. Therefore, initial steps of tympanic ring formation in *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos resemble those of *Hoxa2*$^{-/-}$ single mutants and not wild-type events, in which the ring develops from a single ossification center in the first arch that grows in a circumferential fashion into the second arch (Fig. 7A,B) (Mallo and Gridley, 1996). Similarly, transcripts for the *Cbfa1* gene, an early marker of osteoblast differentiation, are detected in the second arches of E11.5 *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos (Fig. 8C), a typical characteristic of *Hoxa2*$^{-/-}$ single mutants but not of wild-type embryos (Fig. 8A,B) (Kanzler et al., 1998).

The above results indicate that, although only one ring is detected in newborn *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ animals, this cannot be considered as a true phenotypic reversal. Nonetheless, it is clear that development of this structure in *Hoxa2*$^{-/-}$ mutants is influenced by the presence or absence of *Ptx1*. In *Hoxa2*$^{-/-}$ mutants, the two rings have a different size, the duplicated being always smaller, and their distal extremities never fuse. In *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos, the two rings develop in a symmetrical fashion until they eventually fuse. Considering the *Ptx1*$^{-/-}$ mutant phenotype the presence or absence of this gene could eventually account for the differences in growth and shape of the second arch-derived tympanic ring, but cannot easily explain the fusion or not of the rings. One possible explanation for this is that it is somehow related to alterations in the external acoustic meatus (EAM), because its development is closely associated with that of the tympanic ring (Mallo and Gridley, 1996). Histological analyses of *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos revealed the existence of a single EAM associated to the whole length of the tympanic ring as part of a very normal-looking tympanic membrane (Fig. 7F,I). This is reminiscent of what is observed in wild-type embryos

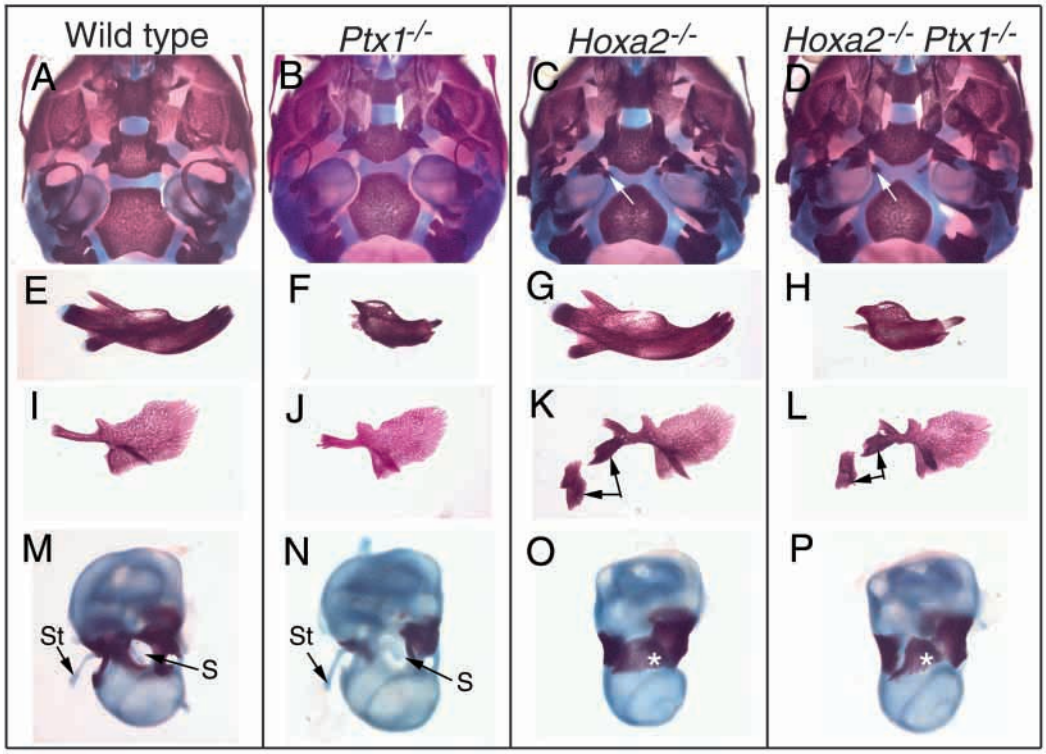

Fig. 6. Skeletal phenotype of *Hoxa2*$^{-/-}$, *Ptx1*$^{-/-}$ single and *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ double mutants. The base of the cranium (A-D), mandible (E-H), squamous bone (I-L) and otic vesicle (M-P) of wild type (A,E,I,M), *Ptx1*$^{-/-}$ (B,F,J,N), *Hoxa2*$^{-/-}$ (C,G,K,O) and *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ (D,H,L,P) mutants are shown. In the base of the cranium of *Hoxa2*$^{-/-}$ and *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos, an extra element associated with the basisphenoid was observed (white arrow in C,D). The mandibles of *Ptx1*$^{-/-}$ and *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos were smaller (F,H). The squamous bone of *Hoxa2* and *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos showed an extra element (arrows in K,L). The styloid process (St) and the stapes (S) (which sits in the oval window) were not present in the *Hoxa2*$^{-/-}$ and *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos (white asterisks in O and P). At least four embryos corresponding to each genotype have been analyzed and showed similar phenotypes.

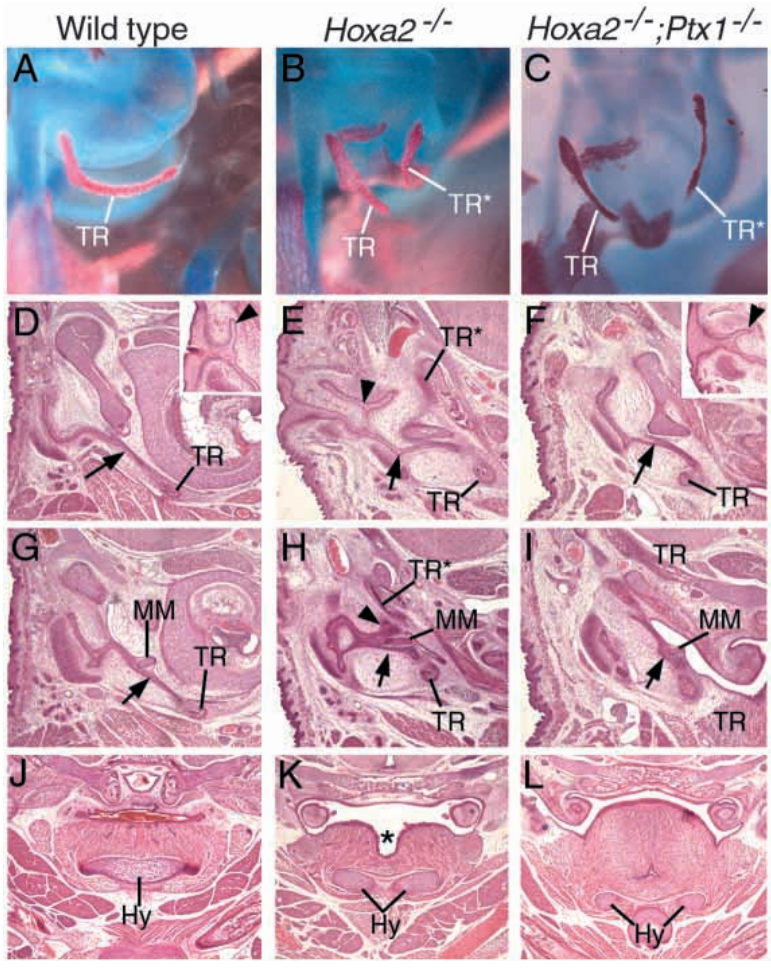

Fig. 7. *Hoxa2*$^{-/-}$ phenotypic rescue in *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ mutants. (A-C) Skeletal preparations of E16.5 wild-type (A), *Hoxa2*$^{-/-}$ (B) and *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ (C) embryos to show the tympanic ring (TR), which is duplicated (TR*) in *Hoxa2*$^{-/-}$ and *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ mutant embryos. (D-I) Histological analysis of the ear region of wild-type (D,G), *Hoxa2*$^{-/-}$(E,H) and *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ (F,I) newborns. Wild-type and *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ double mutants have only one EAM (arrow) reaching to the tympanic ring (TR). *Hoxa2*$^{-/-}$ mutants have an additional EAM (arrowhead) associated with the duplicated ring (TR*). The insets in D and F show a small blind extension (arrowhead) close to the EAM. (G) The tympanic membrane is build up from the EAM (arrow), and epithelium of the middle ear cavity, which entrap the manubrium of the malleus (MM). (H) In *Hoxa2*$^{-/-}$ embryos, the tympanic membrane is distorted by the presence of the two EAMs (arrow and arrowhead). (I) The appearance of the tympanic membrane is quite normal in *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ mutants. (J-L) Tongue phenotype of wild-type (J), *Hoxa2*$^{-/-}$ (K) and *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ (L) newborns. In *Hoxa2*$^{-/-}$ mutants, there is a medial cleft (asterisk), which is lost in the double mutant. Note that the sections are at slightly different levels with respect to the hyoid bone (Hy) to show equivalent areas of the tongue. All sections are frontal. In D-H, lateral is towards the left. (D,E) More posterior areas than G,H. At least three embryos corresponding to each genotype have been analyzed and showed similar phenotypes.

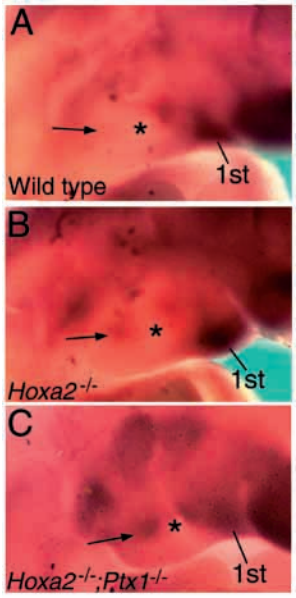

Fig. 8. *Cbfa1* expression in the branchial arches. *Cbfa1* expression was analyzed by in situ hybridization on wild-type (A), *Hoxa2*$^{-/-}$ (B) and *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ (C) E11.5 embryos. The asterisks indicate the location of the external acoustic meatus (in the first pharyngeal cleft). The arrow indicates the extra domain of *Cbfa1* expression in the second arch (not seen in the wild-type embryo). 1st, first branchial arch.

(Fig. 7D,G) but not in *Hoxa2*$^{-/-}$ embryos, which contain a duplicated EAM (Fig. 7E,H) (Mallo and Gridley, 1996). In *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos, a small invagination in the cleft into the second branchial arch can still be detected (Fig. 7F, inset). However, in contrast to *Hoxa2*$^{-/-}$ embryos, in which this invagination deepens into the mesenchyme and becomes associated to the duplicated ring (Fig. 7E), in *Hoxa2*;*Ptx1* double mutants the second arch-associated cleft invagination remains superficial and only the one pointing into the first arch seems to become associated with both ring primordia (Fig. 7F; not shown). Interestingly, careful examination of wild-type embryos revealed the existence of a small and superficial cleft invagination close to the true EAM, reasonably similar to that observed in *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos (Fig. 7D, inset). These results indicate that in the absence of *Ptx1*, development of the EAM in *Hoxa2*-null embryos resembles that of wild-type and not of *Hoxa2*$^{-/-}$;*Ptx1*$^{+/+}$ embryos. It also seems very probable that the fusion of the two tympanic ring primordia observed in *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ newborns is secondary to the EAM phenotype.

Further histological analyses of *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos revealed the disappearance of another *Hoxa2*$^{-/-}$ phenotype in soft tissues. Specifically, the posterior part of the tongue does not show the medial cleft typically present in *Hoxa2* single mutants (Fig. 7K,L). In addition, the styloglossus, which shows a medial trajectory in *Hoxa2*$^{-/-}$ mutants, runs more laterally in *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos, resembling its wild-type trajectory (Fig. 7J-L).

These results, taken together, demonstrate that the upregulation of *Ptx1* observed in *Hoxa2* single-mutant second branchial arches plays a role in the development of the *Hoxa2* mutant phenotype. Thus, one of the functions of *Hoxa2* in the second arch must be to block activation of *Ptx1*.

DISCUSSION

In this paper we have shown that *Hoxa2* normally blocks activation of *Ptx1* in the second branchial arch. In wild-type embryos, *Ptx1* is expressed in the first but not the second arch (Lanctôt et al., 1997), the latter being the major *Hoxa2* expression domain (Prince and Lumsden, 1994; Nonchev et al., 1996; Mallo, 1997). When *Hoxa2* is not present, *Ptx1* is also detected in the second arch mesenchyme in a pattern closely resembling that seen in the first arch mesenchyme. This upregulation has functional significance because, in *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ double mutants, part of the *Hoxa2*$^{-/-}$ phenotype is reversed. In particular, only one EAM was found in double mutant embryos, instead of the two observed in *Hoxa2* single mutants. In addition, the base of the tongue seemed to have lost the medial cleft characteristic of *Hoxa2*$^{-/-}$ embryos. This limited rescue is consistent with the observed domain of *Ptx1* upregulation being highly localized within the second arch. The rest of the *Hoxa2* mutant phenotype (with the exception of the tympanic ring, see below) is not affected by the absence of *Ptx1*, suggesting that other genes are responsible for the genesis of other duplicated structures in the second arch of *Hoxa2* mutants. We have recently identified four more cDNA clones that show upregulation in different areas of the second arch of *Hoxa2* mutants, with two showing expression patterns consistent with involvement in the duplication of the malleus and incus (N.B., M.C. and M.M., unpublished). The functional relevance of these genes is currently under analysis. *Lhx6* could also play a role in the *Hoxa2* mutant phenotype. However, its expression pattern does not give a clear hint about what this role could be. To answer this question, a mutation in this gene must be generated and introduced into the *Hoxa2* mutant background.

Ptx1 in the *Hoxa2*$^{-/-}$ phenotype

Our results show that the phenotype of *Hoxa2*$^{-/-}$ embryos is modified in the absence of *Ptx1*. Some of the *Hoxa2* mutant characteristics are lost and reverted to wild-type-like structures. The most clearly rescued characteristics seem to involve soft tissues, most particularly the EAM, which is not duplicated anymore, and the dorsal part of the base of the tongue. This is very surprising, particularly in the case of the EAM, because this structure derives from the first branchial cleft ectoderm and upregulation of *Ptx1* expression in the second arch is restricted to the mesenchyme. A variety of embryological and genetic experiments has previously indicated that invagination of the EAM is associated to the development of the tympanic ring (Mallo, 2001). Because in *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos an ectopic tympanic ring primordium is formed in the second arch, the absence of EAM duplication is not simply due to the absence of a duplicated ring. One possibility is that the interactions between the second arch ring primordium and the ectoderm covering the second arch side of the cleft occurs differently in the presence or the absence of *Ptx1*. In this scenario, considering that the second arch ring of *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos seem to be able to interact with the EAM induced at the first arch side of the cleft, the ectoderm of the cleft that covers the second arch should have specific characteristics able to respond differently to *Ptx1*-expressing and non-expressing mesenchyme.

Another possible explanation for the phenotypic rescue of

EAM in *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ double embryos is that the duplicated tympanic ring is formed in a location that favors interaction with the first arch side-derived EAM rather than with its second arch counterpart. This explanation would imply a role for *Ptx1* in the spatial and/or temporal induction of the second arch tympanic ring. Consistent with this hypothesis, the second arch-derived rings seem to be located differently in *Hoxa2*$^{-/-}$ and *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos, and preliminary data from our laboratory indicate that the timing of tympanic ring induction is different in the presence and absence of *Ptx1*. A detailed comparison of the spatiotemporal development of the duplicated tympanic ring in *Hoxa2*$^{-/-}$ and *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos will be required to fully evaluate this possibility.

In *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos, the two ring primordia fuse to form a single tympanic ring, a phenomenon never observed in *Hoxa2* single mutants. One possible explanation is that the two rings of *Hoxa2*$^{-/-}$ embryos have an intrinsic inability to fuse, which requires *Ptx1* expression. Alternatively, this effect may be secondary to the presence or not of a duplicated EAM. The independence of the two rings in *Hoxa2*$^{-/-}$ embryos would derive from the independent growth of the two EAMs. In the case of *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos, if each ring primordia is associated with a different part of the same EAM, when the rings and EAMs complete their growth, the tips of the ring primordia, which are directed by the leading edge of the single EAM, will eventually reach each other and fuse. We regard this explanation as more probable because in *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos the two primordia seem to grow toward each other, whereas in *Hoxa2*$^{-/-}$ embryos they grow in a more parallel fashion.

Finally, the absence of medial cleft in the tongue of *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ embryos indicates another role for *Ptx1* in patterning of non-skeletal tissues. It has been suggested that abnormal insertion and trajectory of the hyoglossus might play a role in the abnormal tongue clefting in *Hoxa2*$^{-/-}$ embryos (Ohnemus et al., 2001). Consistent with this hypothesis, in *Hoxa2*$^{-/-}$;*Ptx1*$^{-/-}$ double mutants, this muscle seems to have a more lateral trajectory than in *Hoxa2* single mutants. The hyoglossus is a second arch mesodermal derivative (Carlson, 1999), so it is conceivable that abnormal upregulation of *Ptx1* in the second arch could negatively affect the behavior of the muscle precursors in this area. *Ptx1* expression in the second arch is expected to occur in neural crest-derived mesenchyme (*Hoxa2* is expressed in crest cells). In this case, the neural crest cells would affect patterning/morphogenetic processes in adjacent non-neural crest-derived tissues, similar to what has been described in avian embryos (Köntges and Lumsden, 1996; Schneider and Helms, 2003). It should be noted, however, that abnormal *Ptx1* expression in the second arch muscle precursors of *Hoxa2*$^{-/-}$ embryos cannot be ruled out from our in situ data.

Hoxa2 and Fgf signaling

A very important finding from this paper is that *Hoxa2* acts, at least in part, by repressing genes that play a role in mesenchymal patterning. This is clear for *Ptx1*, and preliminary results from our laboratory indicate that this might also be the case for other genes involved in the production of the duplicated endochondral structures (N.B., M.C. and M.M., unpublished). The repressive nature of this process implies the existence of a *Hoxa2*-independent activating mechanism for those genes susceptible of a *Hoxa2*-dependent block. The *Hoxa2*-dependent block can occur by direct interaction with the promoter of the gene or by interference with some upstream step in the inducing process. A combination of both is also possible, as has been shown for other genes (Guss et al., 2001). Despite extensive efforts, we have so far been unable to find any evidence for a direct interaction of *Hoxa2* with the *Ptx1* promoter. As these are negative results, we cannot rule out the existence of such an interaction, but, they lead us to favor the alternative hypothesis of *Hoxa2* controlling *Ptx1* (and *Lhx6*) expression by interference with the inducing mechanism.

A variety of data indicates that Fgf signaling is a major component of the inducing mechanism for both genes, making this signaling cascade a prime candidate for the target of *Hoxa2* activity. Both *Ptx1* and *Lhx6* are induced beneath an *Fgf8*-expressing epithelium (Lanctôt et al., 1997; Tucker et al., 1999; St Amand et al., 2000). In addition, not only is the epithelium required for mesenchymal induction of both genes, but this induction can also be mimicked by addition of Fgf-soaked beads (Fig. 2D) (Tucker et al., 1999; St Amand et al., 2000). Moreover, genetic evidence indicates the absolute requirement of Fgf8 for mesenchymal induction of these two genes (Fig. 3) (Trumpp et al., 1999) (A.L. and A.N., unpublished). Finally, activation of *Ptx1* can be specifically blocked by an inhibitor of Fgf receptors. As Fgf signaling seems to be the common feature of *Lhx6* and *Ptx1* induction, it is reasonable to hypothesize that *Hoxa2* interferes with activation of these genes by modulating the activity of these Fgf signals. Consistent with this hypothesis, we have shown that the Fgfr inhibitor is also effective in blocking the *Ptx1* activation observed in *Hoxa2* mutant second arches. Moreover, activation of *Lhx6* in the second arch of *Hoxa2*$^{-/-}$ embryos occurs in an area of strong *Hoxa2* expression adjacent to a *Fgf8* expression domain in the rostral second arch epithelium.

Hoxa2 interference with Fgf signaling could explain the defects in bone development observed in transgenic mice upon *Hoxa2* expression in the first arch and developing skull bones (Kanzler et al., 1998). The mandibular hypoplasia can result from either direct or indirect *Hoxa2* activity on *Ptx1* in the first arch, as this phenotype resembles, to a large extent, that of *Ptx1* mutants (Lanctôt et al., 1999; Szeto et al., 1999). However, in the skull bones, where a direct interaction with *Ptx1* or a similar gene is very unlikely, *Hoxa2* interference with Fgf signaling represents a plausible explanation of the phenotype, especially considering that Fgfs are required for bone development in this area (Iseki et al., 1999; Sarkar et al., 2001).

The key question is therefore how *Hoxa2* interferes with Fgf signaling. It is unlikely that this is achieved by control of the signal itself, as *Fgf8* expression is unaffected in *Hoxa2*$^{-/-}$ embryos. Although other Fgfs could be affected, it seems likely that, at least for *Lhx6* and *Ptx1*, Fgf8 is the main player. Other possibilities involve the components of the signal transduction cascades (Boilly et al., 2000), or other modulators, such as genes of the Spry or Sef families (Niehrs and Meinhardt, 2002). Experiments are currently in progress in our laboratory to address this issue.

Interestingly, it has been reported that Fgf signaling can affect Hox gene expression (Cho and De Robertis, 1990; Kolm and Sive, 1995; Partanen et al., 1998; Trainor et al., 2002). In the branchial arches, exogenous Fgf sources are able to downregulate *Hoxa2* expression (Trainor et al., 2002). This

finding, together with our present and previous data, suggests a feedback mechanism (Fig. 9) that could play an important role in establishing the skeletogenic areas in the second arch, which correspond to those areas of low *Hoxa2* activity (Kanzler et al., 1998). When postmigratory second arch crest cells are exposed to Fgf signals, those cells with high *Hoxa2* content will be refractory to these signals, whereas those expressing *Hoxa2* below a given level will be capable of some response. The initial responses to Fgfs in those cells with low *Hoxa2* contents will have a negative effect on *Hoxa2* expression, which in turn will increase their response to the Fgfs. This generates a feedback loop, eventually resulting in cells with very low (or no) *Hoxa2* expression and high responsiveness to Fgf signals. If *Hoxa2*-expressing and non-expressing cells are able to segregate from each other (M.M., unpublished), this feedback loop will eventually result in areas without *Hoxa2* and with high Fgf competence. As Fgfs promote skeletogenesis in the branchial arch mesenchyme (Moore et al., 2002), it is reasonable to assume that these areas belong to the skeletogenic-competent ones, in agreement with our previous results (Kanzler et al., 1998).

Second arch segmental identity

Genetic analyses indicate that *Hoxa2* is essential for providing the second arch with a specific segmental identity (Gendron-Maguire et al., 1993; Rijli et al., 1993; Barrow and Capecchi, 1999). Whether *Hoxa2* performs this role actively by triggering a second arch specific program or in an indirect fashion by modulating mesenchymal responses to exogenous signals has been a matter of discussion. The data we present support a passive rather than an active role for *Hoxa2* in this process. We have shown that, at least for one functionally relevant downstream gene, *Hoxa2* is required for its inactivation in the second arch, most likely by modulation of the competence of neural crest cells to respond to signals provided by surrounding epithelia. It should be noted that *Ptx1* is not the only gene upregulated in the *Hoxa2* mutant second arches. *Lhx6* is another and in our screen we have found others that are upregulated in these mutant second arches with a pattern consistent with an involvement in early steps of endochondral ossification (N.B., M.C. and M.M., unpublished). Conversely, we have so far been unable to confirm differential expression of any gene downregulated in the absence of *Hoxa2*. Therefore, it seems likely that other phenotypic characteristics observed in the *Hoxa2* mutants will turn out to result mostly from the failure of *Hoxa2* to block activation of other genes in the second arch. Genetic experiments are currently in progress to address this possibility.

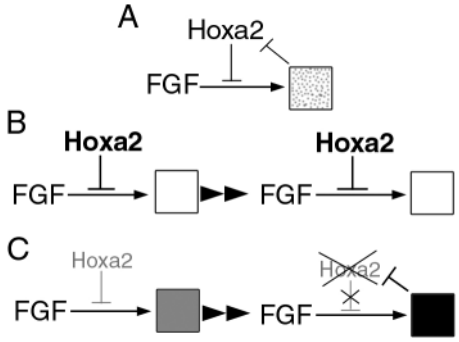

Fig. 9. The relationship between Fgf signaling and *Hoxa2*. (A) Fgfs act on target cells to elicit a response (gray dots in the square). Active Fgf signaling blocks *Hoxa2* expression; conversely, *Hoxa2* blocks Fgf signaling. (B) When neural crest cells expressing high amounts of *Hoxa2* are exposed to Fgfs, no response to the signal is obtained (white box); these cells remain *Hoxa2* positive and Fgf unresponsive. (C) When neural crest cells expressing low (or no) amounts of *Hoxa2* are exposed to Fgfs, responses to the signal are obtained (gray box). These signals will further reduce the *Hoxa2* levels, thus increasing the responsiveness of the cells to Fgfs (black box). These cells will turn *Hoxa2* negative and Fgf responsive.

This interpretation of *Hoxa2* activity is in agreement with previously published data from other laboratories and our own. Expression studies have shown that during normal development both in mouse and chicken embryos, *Hoxa2* is excluded from skeletogenic areas (Kanzler et al., 1998; Grammatopoulos et al., 2000). Conversely, in the *Hoxa2* mutant second arches, skeletal elements develop within the *Hoxa2* expression domain (Kanzler et al., 1998). These results are consistent with the *Hoxa2*-expressing mesenchyme being unable to generate skeletal elements and with the notion that the mutant phenotype results from activation of skeletogenesis in normally silent areas. Importantly, the inability of *Hoxa2*-expressing mesenchyme to give rise to skeletal structures has also recently been reported in chicken embryos after grafting prospective second arch crest into the first branchial arch (Couly et al., 2002). These results argue against the ability of *Hoxa2*-expressing cells to trigger an endogenous second arch-specific skeletogenic program and are consistent with their being unable to respond to skeletogenic signals.

The results of *Hoxa2* misexpression experiments in the first arch of chicken and *Xenopus* embryos have suggested a dominant role of *Hoxa2* in producing second arch structures, with phenotypes that were interpreted as homeotic transformations (Grammatopoulos et al., 2000; Pasqualetti et al., 2000). Conversely, we have shown that activation of *Hoxa2* expression in the first arch of mouse embryos, either in transgenic experiments or by induction with retinoic acid, resulted in deletion of first arch structures without any sign of posterior transformation (Mallo and Brändlin, 1997; Kanzler et al., 1998). The discrepancies between these interpretations could be due to different criteria being used to define the identity of skeletal elements. Alternatively, for *Xenopus* embryos, the discrepancies might be attributed to differences among species, because, in contrast to mouse or chicken, the *Xenopus Hoxa2* homolog seems to be expressed in skeletogenic regions (Pasqualetti et al., 2000). For the chicken experiments, a similar explanation is not plausible as *Hoxa2*-positive cells do not contribute to the skeleton when transplanted to the first arch (Couly et al., 2002).

Implications for a common mechanism of Hox gene function

Hox genes play essential roles in determining segmental identities in different parts of the vertebrate embryo, including the skeletal elements of the paraxial mesoderm and the limbs (Krumlauf, 1994; Zakany and Duboule, 1999; Burke, 2000). So far, there is no clear picture of how Hox genes perform this task, but our findings suggest an interesting explanation. It has been shown that several signaling pathways, including those of Bmps, Fgfs and Hhs, are involved in patterning and

morphogenesis in somitic and limb mesoderm (Pourquie et al., 1996; Oh and Li, 1997; Partanen et al., 1998; Murtaugh et al., 1999; Murtaugh et al., 2001; Pizette and Niswander, 2000). We propose that Hox genes define the competence of these mesenchymal cells to respond to these signals. If each Hox gene has a specific effect on the ability of the mesenchyme to respond (to permit or to block) to one or several of these signaling pathways, and the different Hox genes are able to compete with each other in such activities, a particular Hox combination would result in a specific pattern of response to skeletogenic signals, eventually generating a structure. In this context, the Hox code would be the readout of the responses to these induction processes. Alterations in Hox gene expression would result in altered responses, eventually resulting in abnormal structures, and depending on the particular cases involved, these could be scored as homeotic transformations.

Interestingly, mutations in *Fgfr1* and *Acvr2b* have produced skeletal phenotypes in the vertebrae and limbs similar to those obtained from altered Hox gene expression (Partanen et al., 1998; Oh and Li, 1997). As subtle changes in the expression of some Hox genes were observed, it was suggested that Hox gene expression was under the control of Fgf and Bmp signals, and that the observed phenotypes were secondary to the alterations in Hox gene expression. Based on our results, another (and not mutually exclusive) explanation is possible. If Hox genes modulate Fgf and Bmp signaling, deviations from the normal signaling activities mediated by particular receptors would interfere with the normal readout of the Hox code, eventually producing phenotypic changes. As Fgf activity can affect Hox gene expression (Cho and De Robertis, 1990; Kolm and Sive, 1995; Partanen et al., 1998; Trainor et al., 2002), the abnormal Fgf or Bmp signaling could elicit alterations in expression of specific Hox genes and start a feedback loop, similar to that outlined in Fig. 9, that would eventually potentiate and perpetuate the altered mesenchymal competence to the signals.

We thank Jean Hebert and Susan McConnell for the *Foxg1-cre* mice, Eric Meyers and Gail Martin for the 'Floxed' *Fgf8* mice, Gail Martin for the *Fgf8* probe, Paul Sharpe for the *Lhx6* probe, Christian Wehrle for the help with the Affimetrix microarray procedures, Davor Solter for constant support, and Juan Carlos Izpisúa-Belmonte, António Coutinho and Randy Cassada for reading the manuscript. Research at A.N.'s laboratory is supported by Boehringer Ingelheim and the Wittgenstein Fond, Austria.

REFERENCES

Barrow, J. R. and Capecchi, M. R. (1999). Compensatory defects associated with mutations in *Hoxa1* restore normal palatogenesis to *Hoxa2* mutants. *Development* **126**, 5011-5026.

Boilly, B., Vercoutter-Edouart, A. S., Hondermarck, H., Nurcombe, V. and le Bourhis, X. (2000). FGF signals for cell proliferation and migration through different pathways. *Cytokine Growth Factor Rev.* **11**, 295-302.

Bulfone, A., Kim, H. J., Puelles, L., Porteus, M. H., Grippo, J. F., Rubenstein, J. L. (1993). The mouse *Dlx-2* (*Tes-1*) gene is expressed in spatially restricted domains of the forebrain, face and limbs in midgestation mouse embryos. *Mech. Dev.* **40**, 129-140.

Burke, A. C. (2000). *Hox* genes and the global patterning of the somitic mesoderm. *Curr. Top. Dev. Biol.* **47**, 155-181.

Carlson, B. M. (1999). *Human Embryology and Developmental Biology*. 2nd edn. St Louis, MO: Mosby.

Carroll, S. B. (1995). Homeotic genes and the evolution of arthropods and chordates. *Nature* **376**, 479-485.

Cho, K. W. and de Robertis, E. M. (1990). Differential activation of *Xenopus* homeobox genes by mesoderm-inducing growth factors and retinoic acid. *Genes Dev.* **4**, 1910-1916.

Couly, G., Creuzet, S., Bennaceur, S., Vincent, C. and le Douarin, N. M. (2002). Interactions between *Hox*-negative cephalic neural crest and the foregut endoderm in patterning the facial skeleton in the vertebrate head. *Development* **129**, 1061-1073.

Crossley, P. H. and Martin, G. R. (1995). The mouse *Fgf8* gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* **121**, 439-451.

Gendron-Maguire, M., Mallo, M., Zhang, M. and Gridley, T. (1993). *Hoxa-2* mutant mice exhibit homeotic transformation of skeletal elements derived from cranial neural crest. *Cell* **75**, 1317-1331.

Grammatopoulos, G. A., Bell, E., Toole, L., Lumsden, A. and Tucker, A. S. (2000). Homeotic transformation of branchial arch identity after *Hoxa2* overexpression. *Development* **127**, 5355-5365.

Guss, K. A., Nelson, C. E., Hudson, A., Kraus, M. E. and Carroll, S. B. (2001). Control of a genetic regulatory network by a selector gene. *Science* **292**, 1164-1167.

Hebert, J. M. and McConnell, S. K. (2000). Targeting of cre to the *Foxg1* (*BF-1*) locus mediates *loxP* recombination in the telencephalon and other developing head structures. *Dev. Biol.* **222**, 296-306.

Iseki, S., Wilkie, A. O. and Morriss-Kay, G. M. (1999). Fgfr1 and Fgfr2 have distinct differentiation- and proliferation-related roles in the developing mouse skull vault. *Development* **126**, 5611-5620.

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.

Kanzler, B., Foreman, R. K., Labosky, P. A. and Mallo, M. (2000). BMP signaling is essential for development of skeletogenic and neurogenic cranial neural crest. *Development* **127**, 1095-1104.

Kolm, P. J. and Sive, H. L. (1995). Regulation of the Xenopus labial homeodomain genes, *HoxA1* and *HoxD1*: activation by retinoids and peptide growth factors. *Dev. Biol.* **167**, 34-49.

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

Krumlauf, R. (1994). *Hox* genes in vertebrate development. *Cell* **78**, 191-201.

Lanctôt, C., Lamolet, B. and Drouin, J. (1997). *Ptx1* defines the most anterior domain of the embryo and differentiates posterior from anterior lateral mesoderm. *Development* **124**, 2807-2817.

Lanctôt, C., Moreau, A., Chamberland, M., Tremblay, M. L. and Drouin, J. (1999). Hindlimb patterning and mandible development require the *Ptx1* gene. *Development* **126**, 1805-1810.

Le Douarin, N. M. and Kalcheim, C. (1999). *The Neural Crest*. Cambridge, UK: Cambridge University Press.

Lewis, E. (1978). A gene complex controlling segmentation in *Drosophila*. *Nature* **27**, 565-570.

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. (2001). Formation of the middle ear: recent progress on the developmental and molecular mechanisms. *Dev. Biol.* **231**, 410-419.

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

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., Schrewe, H., Martin, J. F., Olson, E. N. and Ohnemus, S. (2000). Assembling a functional tympanic membrane: signals from the external acoustic meatus coordinate development of the malleal manubrium. *Development* **127**, 4127-4136.

Meyers, E. N., Lewandoski, M. and Martin, G. R. (1998). An *Fgf8* mutant allelic series generated by Cre- and Flp-mediated recombination. *Nat. Genet.* **18**, 136-141.

Mohammadi, M., McMahon, G., Sun, L., Tang, C., Hirth, P., Yeh, B. K., Hubbard, S. R. and Schlessinger, J. (1997). Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. *Science* **276**, 955-960.

Moore, R., Ferretti, P., Copp, A. and Thorogood, P. (2002). Blocking endogenous FGF-2 activity prevents cranial osteogenesis. *Dev. Biol.* **243**, 99-114.

Murtaugh, L. C., Chyung, J. H. and Lassar, A. B. (1999). Sonic hedgehog promotes somitic chondrogenesis by altering the cellular response to BMP signaling. *Genes Dev.* **13**, 225-237.

Murtaugh, L. C., Zeng, L., Chyung, J. H. and Lassar, A. B. (2001). The chick transcriptional repressor *Nkx3.2* acts downstream of Shh to promote BMP-dependent axial chondrogenesis. *Dev. Cell* **1**, 411-422.

Niehrs, C. and Meinhart, H. (2002). Modular feedback. *Nature* **417**, 35-36.

Nonchev, S., Vesque, C., Maconochie, M., Seitanidou, T., Ariza-McNaughton, L., Frain, M., Marshall, H., Sham, M. H., Krumlauf, R. and Charnay, P. (1996). Segmental expression of *Hoxa-2* in the hindbrain is directly regulated by *Krox-20*. *Development* **122**, 543-554.

Oh, S. P. and Li, E. (1997). The signaling pathway mediated by the type IIB activin receptor controls axial patterning and lateral asymetry in the mouse. *Genes Dev.* **11**, 1812-1826.

Ohnemus, S., Bobola, N., Kanzler, B. and Mallo, M. (2001). Different levels of *Hoxa2* are required for particular developmental processes. *Mech. Dev.* **108**, 135-147.

Partanen, J., Schwartz, L. and Rossant, J. (1998). Opposite phenotypes of hypomorphic and Y766 phosphorylation site mutations reveal a function for Fgfr1 in anterioposterior patterning of mouse embryos. *Genes Dev.* **12**, 2332-2344.

Pasqualetti, M., Ori, M., Nardi, I. and Rijli, F. M. (2000). Ectopic *Hoxa2* induction after neural crest migration results in homeosis of jaw elements in Xenopus. *Development* **127**, 5367-5378.

Pizette, S. and Niswander, L. (2000). BMPs are required at two steps of limb chondrogenesis: formation of prechondrogenic condensations and their differentiation into chondrocytes. *Dev. Biol.* **219**, 237-249.

Prince, V. and Lumsden, A. (1994). *Hoxa-2* expression in normal and transposed rhombomeres: independent regulation in the neural tube and neural crest. *Development* **120**, 911-923.

Pourquie, O., Fan, C. M., Coltey, M., Hirsinger, E., Watanabe, Y., Breant, C., Francis-West, P., Brickell, P., Tessier-Lavigne, M. and le Douarin, N. M. (1996). Lateral and axial signals involved in avian somite patterning: a role for BMP4. *Cell* **84**, 461-471.

Rijli, F. M., Mark, M., Lakkaraju, S., Dierich, A., Dolle, P. and Chambon, P. (1993). A homeotic transformation is generated in the rostral branchial region of the head by disruption of *Hoxa-2*, which acts as a selector gene. *Cell* **75**, 1333-1349.

Sarkar, S., Petiot, A., Copp, A. J., Ferretti, P. and Thorogood, P. (2001). FGF-2 promotes skeletogenic differentiation of cranial neural crest cells. *Development* **128**, 2143-2152.

Schneider, R. A. and Helms, J. A. (2003). The cellular and molecular origins of beak morphology. *Science* **299**, 565-568.

Serbedzija, G. N., Bronner-Fraser, M. and Fraser, S. E. (1992). Vital dye analysis of cranial neural crest cell migration in the mouse embryo. *Development* **116**, 297-307.

St Amand, T. R., Zhang, Y., Semina, E. V., Zhao, X., Hu, Y. P., Nguyen, L., Murray, J. C. and Chen, Y. P. (2000). Antagonistic signals between BMP4 and FGF8 define the expression of *Pitx1* and *Pitx2* in mouse tooth-forming anlage. *Dev. Biol.* **217**, 323-332.

Szeto, D. P., Rodriguez-Esteban, C., Ryan, A. K., O'Connell, S. M., Liu, F., Kioussi, C., Gleiberman, A. S., Izpisúa-Belmonte, J. C. and Rosenfeld, M. G. (1999). Role of the Bicoid-related homeodomain factor Pitx1 in specifying hindlimb morphogenesis and pituitary development. *Genes Dev.* **13**, 484-494.

Thesleff, I., Vaahtokari, A. and Partanen, A. M. (1995). Regulation of organogenesis. Common molecular mechanisms regulating the development of teeth and other organs. *Int. J. Dev. Biol.* **39**, 35-50.

Trainor, P. A. and Krumlauf, R. (2001). *Hox* genes, neural crest cells and branchial arch patterning. *Curr. Opin. Cell. Biol.* **13**, 698-705.

Trainor, P. A., Ariza-McNaughton, L. and Krumlauf, R. (2002). Role of the isthmus and FGFs in resolving the paradox of neural crest plasticity and prepatterning. *Science* **295**, 1288-1291.

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.

Tucker, A. S., Yamada, G., Grigoriou, M., Pachnis, V. and Sharpe, P. T. (1999). Fgf-8 determines rostral-caudal polarity in the first branchial arch. *Development* **126**, 51-61.

Wang, W., Chan, E. K., Baron, S., van de Water, T. and Lufkin, T. (2001). *Hmx2* homeobox gene control of murine vestibular morphogenesis. *Development* **128**, 5017-5029.

Zakany, J. and Duboule, D. (1999). *Hox* genes in digit development and evolution. *Cell Tissue Res.* **296**, 19-25.