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

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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 is 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
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 (Coulby 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; Coulby 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 Capechi, 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 (Ptx1 – 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**

**Affimex 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 Affimex U74A microarrays as recommended by the manufacturer.

**Mutant and transgenic animals and embryos**

The Hoxa2 (Gendron-Maguire et al., 1993) and Ptx1 (Lancetò et al., 1999) mutant strains have been described previously. The Fg8;Foxg1-cre mutant embryos were created by intercrosses of Fg8floxflox females and Fg8–/–;Foxg1cre/+ 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. Fg8 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% CO2/95% 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 (Lancetò et al., 1997), Chba (Kanzler et al., 1998), Fg8 (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).

**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 Affimexir 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 (Lancôt 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) (Lancôt 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) (Lancôt 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.

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 epithelium (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 epidermis. 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

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**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 (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.

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[The text continues with further details and discussion related to the figure, gene expression patterns, and experimental methods.]
**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,L), 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 PBS. (F) Wild-type first and second arches incubated with epithelium in the presence of a bead soaked in 13.5 mM SU5402. (G) Wild-type first and second arches incubated with epithelium in the presence of a bead soaked in 13.5 mM SU5402. (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. 2I). 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.

**Partial rescue of the Hoxa2+/– phenotype in Hoxa2+/–;Ptx1+/– embryos**

The above results indicate that in normal embryos Hoxa2

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).

**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 (Lancôt 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 basi-phenoid 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 extradermal element associated with Meckel’s cartilage (Lancôt et al., 1999), also seemed unaffected by the Hoxa2 mutation (Fig. 5C,D; Fig. 6E,F), and the hindlimb phenotype of Hoxa2+/–;Ptx1+/– embryos resembled that of their Ptx1+/– littermates (Lancôt et al., 1999; Szeto et al., 1999).

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 Cbfal 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. 5.** Middle ear skeletal phenotype of Hoxa2+/–, Ptx1+/– and Hoxa2+/–;Ptx1+/– mutants. Stained middle ear skeletal elements from wild-type (A), Hoxa2+/– (B), Ptx1+/– (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.
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,L,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.
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 (Lancétő 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 primordium (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 primordium is associated with a different part of the same EAM, when the rings and EAMs complete their growth, the tips of the ring primordium, 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 (Ohmemus 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**

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 Sry 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.

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

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.
morphogenesis in somitic and limb mesoderm (Pourquié 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 Aevr2b 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.

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