# *Hoxa2* downregulates *Six2* in the neural crest-derived mesenchyme

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## Summary

**The Hoxa2 transcription factor acts during development of the second branchial arch. As for most of the developmental processes controlled by Hox proteins, the mechanism by which Hoxa2 regulates the morphology of second branchial arch derivatives is unclear. We show that Six2, another transcription factor, is genetically downstream of Hoxa2. High levels of *Six2* are observed in the *Hoxa2* loss-of-function mutant. By using a transgenic approach to overexpress *Six2* in the embryonic area controlled by *Hoxa2*, we observed a phenotype that is reminiscent of the *Hoxa2* mutant phenotype. Furthermore, we demonstrate that Hoxa2 regulation of *Six2* is confined to a 0.9 kb fragment of the *Six2* promoter and that Hoxa2 binds to this promoter region. These results strongly suggest that *Six2* is a direct target of Hoxa2.**

Key words: *Hoxa2*, *Six2*, Hox genes, Branchial arches, Neural crest, Mouse

## Introduction

The facial skeleton is formed by neural crest cells (Le Douarin and Kalcheim, 1999). Its development can be summarized in three main steps: cranial neural crest cells are produced in the developing neural tube, they migrate to the ventral region of the head (frontonasal mass and branchial arches), and, at the endpoint of migration, they form the different skeletal elements, each with a precise shape and in a precise position (reviewed by Chambers and McGonnell, 2002; Gammil and Bronner-Fraser, 2003; Helms and Schneider, 2003; Santagati and Rijli, 2003). As the neural crest is a dynamic population, a central issue in craniofacial development has been where these cells receive the patterning information to forge the different skeletal elements. Recent experimental observations indicate that the cranial neural crest is not pre-patterned before migration, but rather migrates as a naïve population. The epithelia, in particular the foregut endoderm, produce signals received by the neural crest at its final destinations, and these are interpreted to form the different skeletal elements (Couly et al., 2002).

Hox genes are key developmental regulators required to specify segmental identity in the developing embryo (Carroll, 1995). Genetic analyses in flies and vertebrates have yielded extensive knowledge about the developmental processes regulated by Hox genes, but the molecular events directly controlled by these genes are still largely unknown (for reviews, see Krumlauf, 1994; Zakany and Duboule, 1999; Burke, 2000; Trainor and Krumlauf, 2001; Alonso, 2002).

Hox-negative and Hox-positive domains can be distinguished in the cranial neural crest (Prince and Lumsden, 1994; Graphin-Bottom et al., 1995; Couly et al., 1996; Köntges and Lumsden, 1996). The visceral skeleton of the face derives from the Hox-negative first branchial arch, with a very limited contribution from the more posterior Hox-positive branchial arches. Moreover, ectopic expression of Hox genes in anterior Hox-negative crest cells in chicken (Creuzet et al., 2002) results in the absence of the facial skeleton. A similar effect is observed in mouse, following *Hoxa2* overexpression in the head mesenchyme (Kanzler et al., 1998).

Among Hox genes, *Hoxa2*, together with *Hoxb2*, shows the most anterior domain of expression in the cranial neural crest, corresponding to the population that migrates to the second branchial arch (Prince and Lumsden, 1994; Nonchev et al., 1996; Mallo, 1997). In mouse, disruption of the *Hoxa2* gene mainly affects second branchial arch development. Second arch skeletal elements (stapes, styloid process, lesser horn of the hyoid bone) are transformed into first arch-specific skeletal elements (incus, malleus and tympanic ring), arranged in a mirror image disposition to their first arch counterparts (Gendron-Maguire et al., 1993; Rijli et al., 1993; Barrow and Capecchi, 1999). This arrangement suggests a common source of information located between the first and second branchial arch (Rijli et al., 1993; Mallo and Brandlin, 1997), with a different interpretation of this common signal in neural crest cells expressing *Hoxa2*. More specifically, *Hoxa2* is thought to interfere negatively with the response of the neural crest cells to skeletogenic cues (Couly et al., 2002; Bobola et al., 2003); accordingly, chondrogenesis in the second arch takes place exclusively in areas that are free of *Hoxa2* expression (Kanzler et al., 1998). Thus, *Hoxa2* patterns the second arch skeleton by limiting its formation. Consistent with a role in controlling the size of the condensations, overexpression of *Hoxa2* in chick and in frog confers a late, postmigratory, patterning role to *Hoxa2* during development of the branchial arch-derived skeleton (Grammatopoulos et al., 2000; Pasqualetti et al., 2000).

Because of the negative effect on the formation of the facial skeleton, the absence of Hox gene expression in the anterior part of the embryo has been proposed as a crucial factor to

allow the evolution of the head and lower jaw in gnathostomes (Creuzet, 2002; Manzanares and Nieto, 2003). As with most developmental processes regulated by Hox genes, the molecular cascade initiated by these genes to culminate in the inhibition of craniofacial skeletogenesis is unknown.

In a subtraction approach, designed to clarify the molecular cascades initiated by Hoxa2 to control skeletogenesis in the second branchial arch (Bobola et al., 2003), we have found *Six2* as a gene regulated by Hoxa2.

The Six family of homeobox transcription factors, characterized by a Six domain and a homeodomain, counts six members in mammals (Kawakami et al., 2000). Members of this family share transcriptional properties and the ability to interact physically and functionally with Eya proteins, both in *Drosophila* and in vertebrates (Bonini et al., 1997; Pignoni et al., 1997; Heanue et al., 1999; Ohto et al., 1999; Ikeda et al., 2002; Ozaki et al., 2002; Li et al., 2003; Ruf et al., 2004). Six genes differ largely in their expression pattern during embryogenesis, and gene inactivation experiments have revealed that these genes control a variety of developmental processes (Klesert et al., 2000; Carl et al., 2002; Li et al., 2002; Laclef et al., 2003a; Laclef et al., 2003b; Lagutin et al., 2003; Xu et al., 2003; Zheng et al., 2003; Ozaki et al., 2004).

*Six2* function has not yet been characterized. Its expression is restricted to the head mesenchyme, foregut, stomach, kidney and genital tubercle (Oliver et al., 1995). Inactivation of *Six1*, its closest homolog, affects muscle, kidney, branchial arch derivatives and inner ear development (Laclef et al., 2003a; Laclef et al., 2003b; Xu et al., 2003; Zheng et al., 2003; Ozaki et al., 2004). The presence of incomplete and/or smaller cartilages and bones characterizes the craniofacial phenotype of *Six1* null mice (Laclef et al., 2003b). Indeed, *Six1* inactivation provided the first direct evidence implicating Six genes in the development of the facial skeleton; the strong craniofacial phenotype of the *Eya1* mutant and the reported genetic interaction of *Eya* and Six genes give additional, indirect hints (Xu et al., 1999).

Here we show that *Hoxa2* negatively regulates *Six2* expression during second arch development. Ectopic expression of *Six2* in the second branchial arch causes distinctive phenotypic features seen in the *Hoxa2* mutant. Furthermore, gain-of-function experiments show that Hoxa2 is sufficient to downregulate *Six2* expression in the neural-crest-derived mesenchyme. We also show that a 0.9 kb fragment of the *Six2* promoter is the target of Hoxa2 regulation and that Hoxa2 physically interacts with the proximal region of this promoter.

Together, our results show that Six2 is genetically downstream of Hoxa2 in the second branchial arch and suggest that regulation of *Six2* may be one of the mechanisms utilized by Hoxa2 to pattern the second arch skeleton.

Finally, we propose that the *Six2* gene could be a target of Hox proteins in different developmental processes in addition to patterning the second arch.

## Materials and methods

### Molecular and phenotypic analyses

In situ hybridization was performed as described (Kanzler et al., 1998), using *Six2* (Oliver et al., 1995), *Hoxa2* (Mallo, 1997) and *Sox9* (Kanzler et al., 1998) probes. Embryonic day (E) 10.5 *Msx2-Hoxa2* transgenic and control embryos were cut in half, and each half hybridized with a *Hoxa2* or *Six2* probe to allow direct comparison. Skeletal preparations were previously described (Mallo and Brändlin, 1997).

### RT-PCR

Second arches of E10.5 embryos from *Hoxa2*$^{+/-}$ intercrosses and *a2-Six2* transgenics were dissected, frozen and pooled according to genotype. RT-PCR was performed using Superscript Preamplification System (Invitrogen) and Taq polymerase (PeqLab) with the following primers: Six2F 5′-CAGCCGCCACCATGTCCATGCTG-3′; Six2R 5′-GAACTTGCGCCGCACGCGGTAC-3′; Six1F 5′-AAGAACCGGAG-GCAAAGAGACC-3′; Six1R 5′-CCAATATCTCCCCACTTAGGAA-CC-3′; Six4F 5′-AACCAGTATGGCATTGTCCAGATCC-3′; Six4R 5′-ACTGCAGAACCAAGCGCTGTTCTC-3′; Six5F 5′-GAGTGACT-GCGCTGCAACTTCCCTCG-3′; Six5R 5′-AGGGCTCCTCCACG-GGTACCGAC-3′; GadphF 5′-ACCACAGTCCATGCCATCAC-3′; GadphR 5′-TCCACCACCCTGTTGCTGTA-3′; Hoxa2F 5′-GCCT-GAGTATCCCTGGATG-3′; Hoxa2R 5′-ACCCTTCCCTCTCCAG-AAG-3′. First-strand cDNA was subjected to 24 amplification cycles. The specificity of each PCR product was confirmed by sequencing.

### Mutant and transgenic animals and embryos

*Hoxa2* mutant mice were described (Gendron-Maguire et al., 1993). Transgenic embryos were generated by pronuclear injection of the following transgenes: *a2-Six2*, containing *Six2* cDNA, amplified with primers Six2F 5′-CAGCCGCCACCATGTCCATGCTG-3′ Six2R 5′-CTCTAGGAGCCCAGGTCCACAAGG-3′ cloned downstream the *Hoxa2* enhancer (Kanzler et al., 1998); *Msx2-Hoxa2* (Kanzler et al., 1998); *900Six2-lacZ* containing *Bam*HI (–893) – Sph1 (+18) *Six2* promoter fragment obtained by screening the RPCI mouse PAC library 21 (Osoegawa et al., 2000) (provided by UK HGMP Resource Centre), cloned into pCMVbeta (Clontech). *900Six2-lacZ* was injected to generate both transgenic embryos and transgenic lines. A line showing high expression in the first branchial arch was crossed to *Hoxa2*$^{+/-}$ mice to obtain *900Six2-lacZ*; *Hoxa2*$^{+/-}$, which were mated to *Hoxa2*$^{+/-}$ to generate *900Six2-lacZ*;*Hoxa2*$^{-/-}$ embryos.

### In vitro transcription/translation and electrophoretic mobility shift assay

Mouse Hoxa2 cDNA, containing a HA tag inserted in frame before the stop codon, mouse Meis1 cDNA and human Pbx1a cDNA (Di Rocco et al., 1997) were cloned in pcDNA3 (Invitrogen) and transcribed/translated using T7-coupled TNT rabbit reticulocytes (Promega).

*Bst*EII/*Ssp*I probe, probe 1 and probe 2 were labeled with $\alpha^{32}$P-dCTP. The binding reaction was performed as described (Scheidereit, 1987).

The sequence of the oligonucleotides used as probes and competitors is shown in Fig. 6. For the supershift experiments, 40 ng of anti-HA antibodies (rat monoclonal 3F10, Roche) were added to the reaction.

## Results

### *Six2* is upregulated in the *Hoxa2* mutant

The Hoxa2 transcription factor is essential for the development of the second branchial arch, but its mechanism of action is largely unknown. To identify target genes of Hoxa2, we analyzed the expression profiles of wild-type and *Hoxa2* mutant second branchial arches (Bobola et al., 2003). Among the differentially expressed clones found in our screen, the mRNA corresponding to Six2 showed a threefold increase in the absence of Hoxa2.

*Hoxa2* is expressed in neural crest cells migrating from rhombomere 4 to populate the second arch, later its main expression domain (Prince and Lumsden, 1994; Nonchev et al., 1996; Mallo, 1997). *Six2* expression in the branchial area is first detected at E9.5, predominantly in the first arch (Oliver et al., 1995). At this stage, no obvious differences in *Six2* expression pattern could be observed between wild-type and *Hoxa2* mutant

second arches (not shown). At E10.5, *Six2* expression was restricted to the posterior area of the second branchial arch. In addition, a second, more proximal domain of *Six2* expression appeared in the mutant second arch (Fig. 1A,D, arrow). The spatial and temporal coordinates of *Six2* expression in the mutant second arch, together with the signal intensity, were equivalent to those of the *Six2* expression domain in the first arch mesenchyme. In *Hoxa2* mutant embryos, the first and second arch *Six2*-expressing areas were symmetrical with respect to the first branchial cleft (Fig. 1A,D).

At E11.5, *Six2* was mainly expressed in the first arch with a characteristic pattern; a small restricted area in the second arch was positive for *Six2* (Fig. 1B, arrowhead). At this stage, the *Six2* expression domain in the mutant second arch still appeared as a duplication of the first arch expression domain (Fig. 1E). In addition, a strong ectopic domain of *Six2* expression was detected in the mutant mesenchyme proximal to the second branchial arch and delimited externally by the forming otic capsule (Fig. 1E, white arrow). One day later, the first and second branchial arch had completely fused. The first cleft was still evident and the pattern of *Six2* expression around the cleft was essentially unchanged. However, an increase in *Six2* expression in the mutant could be observed in the area surrounding the otic capsule (Fig. 1C,F, white arrow).

In the absence of *Hoxa2*, a duplicated incus and malleus form in the second branchial arch. To see if *Six2* upregulation in the second arch was spatially associated with the ectopic cartilaginous condensations, we examined mutant and wild-type embryos for the expression of the chondrogenic marker *Sox9* (Ng et al., 1997; Zhao et al., 1997). In E12.5 wild-type embryos, *Sox9* mRNA was mainly expressed in the area derived from the first branchial arch, consistent with the restricted chondrogenesis that takes place in the second branchial arch (Fig. 1G). In the *Hoxa2* mutant, an equivalent *Sox9* expression in the first and second arch anticipated the formation of incus and malleus in the first arch and their duplicated counterparts in the second arch (Fig. 1H). *Six2* signal could be detected in close proximity to *Sox9* (Fig. 1C,D). In a more detailed inspection of the mutual localization of *Six2* and *Sox9* mRNAs, performed by in-situ hybridization on adjacent sections of branchial arches of E11.5 *Hoxa2* mutant embryos, a defined mesenchymal area, including the first and second branchial arch, was positive for both *Six2* and *Sox9* (Fig. 1I,L, arrows). We conclude that the upregulation of *Six2* takes place in the area fated to give rise to the skeletal duplication in the *Hoxa2* mutant.

In addition to *Six2*, the genes *Six1*, *Six4* and *Six5* are also expressed in the branchial area (Oliver et al., 1995; Klesert et al., 2000; Ozaki et al., 2001). To distinguish if the upregulation of *Six2* observed in the *Hoxa2* mutant second arch is specifically restricted to *Six2* or if it is a common feature shared by the other Six genes, we performed semiquantitative RT-PCR on total RNA extracted from E10.5 *Hoxa2* mutant and wild-type arches, using *Six1*-, *Six2*-, *Six4*- and *Six5*-specific primers. As expected, we observed a significant increase in *Six2* signal in the mutant versus wild-type second arch, but *Six1*, *Six4* and *Six5* levels (as well as *Gadph* control) remained unaffected (Fig. 2). These data indicate that *Hoxa2* negatively controls *Six2* expression in the second branchial arch specifically and does not affect any of the other Six genes expressed in this area.

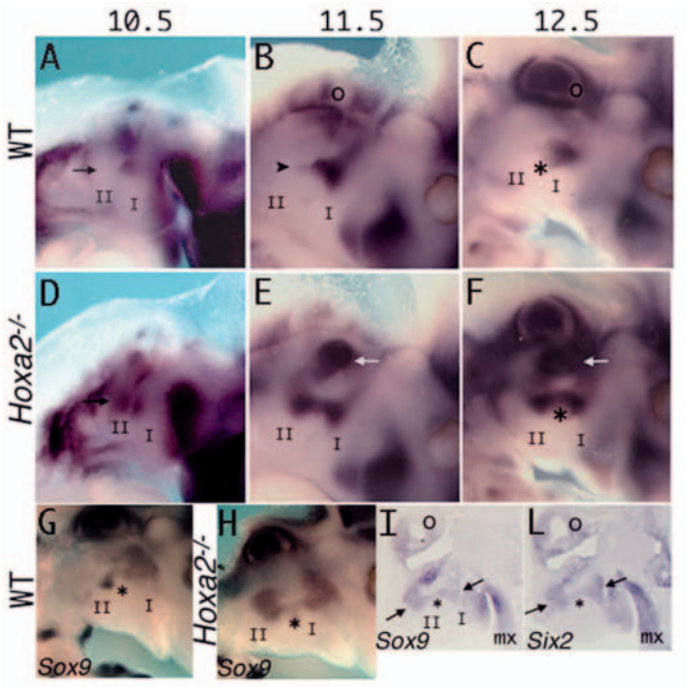


**Fig. 1.** *Six2* is upregulated in the second branchial arch of *Hoxa2*$^{-/-}$ embryos. Whole-mount in-situ hybridization of wild-type (A-C) and *Hoxa2*$^{-/-}$ (D-F) embryos. (A,D) E10.5 embryos. *Six2* is detected in the *Hoxa2* mutant second arch (arrow) in a symmetrical location to its first arch counterpart. (B,E) E11.5 embryos. A small area expressing *Six2* is visible in the wild-type second arch (arrowhead). The mutant second arch is equivalent to the first arch in terms of *Six2* expression; ectopic *Six2* expression is also detected close to the developing otic vesicle (white arrow). (C,F) E12.5 embryos. In the mutant, *Six2* signal is increased around the otic vesicle (white arrow). (G,H) Expression of *Sox9* in E12.5 wild-type (G) and mutant (H) arches marking the position of the cartilaginous condensations. Note the duplicated pattern in the mutant and its position relative to the first branchial cleft (asterisk). (I,L) *Sox9* (I) and *Six2* (L) probes were hybridized to adjacent sections of E11.5 branchial arches of the *Hoxa2* mutant. Arrows indicate the expression domains of *Sox9* and *Six2* in the first arch and their duplication in the second arch. I, first arch; II, second arch; mx: maxilla; o: otic vesicle; asterisk: first branchial cleft.

## *Six2* acts functionally downstream of Hoxa2 in the second branchial arch

In the absence of *Hoxa2*, *Six2* is expressed in the proximal domain of the second arch, symmetrically to the *Six2* expression domain in the first arch. Duplicated first arch skeletal elements arise in the very same area in the *Hoxa2* mutant (Fig. 3A,B)

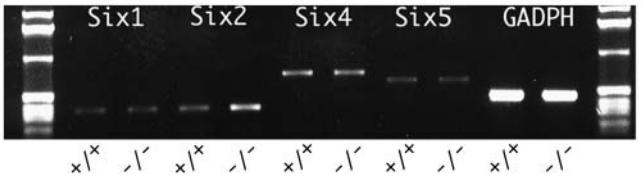


**Fig. 2.** The expression of other Six family members is not affected by *Hoxa2*. Semi-quantitative RT-PCR on RNA extracted from E10.5 second branchial arches of wild-type (+/+) and *Hoxa2*$^{-/-}$ (–/–) embryos using specific primers for *Six1*, *Six2*, *Six4*, *Six5* and *GADPH*. The results shown were observed in three independent experiments.

(Gendron-Maguire et al., 1993; Rijli et al., 1993; Barrow and Capecchi, 1999).

To test if these two events could be associated, namely if *Six2* upregulation might be one of the causative events in the generation of the *Hoxa2* mutant phenotype, we overexpressed *Six2* cDNA in the second branchial arch of wild-type embryos using a *Hoxa2* enhancer (Nonchev et al., 1996). In-situ hybridization on E10.5 transgenic embryos (*a2-Six2*) showed that *Six2* mRNA was additionally detected in the second and more posterior branchial arches. Strong ectopic *Six2* expression was also observed in the somitic mesoderm (Fig. 3C,D).

We then analyzed the effects of *Six2* overexpression on the skeletal phenotype of E18.5 *a2-Six2* embryos. The second arch skeleton is composed of three cartilages (stapes, styloid process and lesser horn of the hyoid bone), which are transformed into different skeletal elements in the *Hoxa2* mutant. All these elements displayed morphological changes in transgenic embryos overexpressing *Six2* (Fig. 3E,F,H,I).

The stapes, which is lost in the *Hoxa2* mutant, was either reduced or absent in *a2-Six2* transgenics (2/6). An ectopic cartilage, fused to the proximal part of the styloid process, extended into the oval window and in front of the incus. This cartilage, which in shape and position resembled a stapedial arch, may be an intermediate between loss of the stapes and formation of a duplicated incus, as observed in the *Hoxa2* mutant (4/6) (Fig. 3E,F). The styloid process was thicker (Fig. 3E,F) or abnormally elongated and fused to the lesser horn of the hyoid bone, forming a long Meckel-like cartilage (2/6) (Fig. 3H,I). The lesser horn, absent in the *Hoxa2* mutant, was misshapen, enlarged and fused to the greater horn (6/6); in most cases, it appeared as a bifurcation of the greater horn. By contrast to the noticeable effects in cartilages, bone formation was relatively unchanged and in only one case tympanic ring growth was mildly affected (Fig. 3H,I).

Molecular analysis of *a2-Six2* transgenic embryos showed no noticeable effect of overexpression of *Six2* on the levels of *Hoxa2* in the second branchial arch (Fig. 3J), thus ruling out *Hoxa2* downregulation as a possible cause of the transgenic phenotype.

Compared with the *Hoxa2* phenotype, we observed two main defects: one was as featured in the *Hoxa2* mutant (absent stapes, incus duplication), while the other consisted of enlargements of the elements patterned by *Hoxa2* (lesser horn, styloid process abnormally elongated). In all cases, overexpression of *Six2* in the second branchial arch resulted in the formation of ectopic cartilage, one of the phenotypic characteristics of the *Hoxa2* mutant (Kanzler et al., 1998).

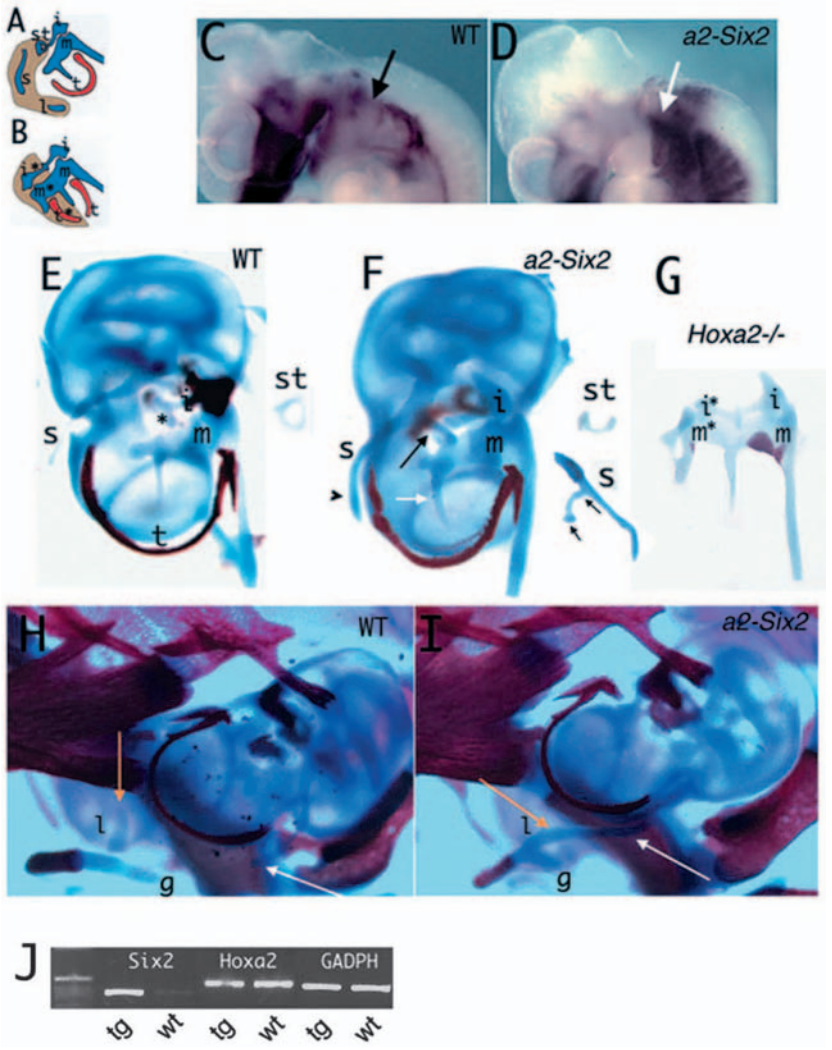


**Fig. 3.** Middle ear skeletal phenotype of *a2-Six2* transgenics. (A) Schematic representation of the second (brown background) and first-arch-derived skeleton. (B) In the absence of *Hoxa2*, duplicated first arch elements derive from the second arch (brown). (C) Expression of *Six2* in E10.5 wild-type embryos. *Six2* mRNA is almost excluded from the second (black arrow) and more posterior arches. (D) *Six2* is ectopically expressed in the second (white arrow) and more posterior arches and in the somitic mesoderm of *a2-Six2* embryos. (E) Middle ear skeleton of an E18.5 wild-type embryo. The stapes is shown in the oval window (*) and after dissection. (F) Middle ear skeleton of a transgenic littermate. An ectopic cartilage, connected to the styloid process, extends to face the incus (black arrow); ventral view of the dissected styloid process, with the ectopic cartilage delimited by arrows, is shown on the right. The styloid process is thicker (arrowhead) and the manubrium of the malleus is curved (white arrow). The stapes, dissected and shown on the right, is incomplete. Malformation of the tympanic ring was observed only once. (G) Mirror image cartilages in the Hoxa2 mutant. Duplicated elements are marked with an asterisk. (H) Lateral view of a wild-type skull: orange arrow indicates the distal extremity of the lesser horn of the hyoid bone; the end of the styloid process is marked by a white arrow, the greater horn is also indicated. (I) In transgenic embryos, the lesser horn elongates and fuses to the styloid process, generating a continuous structure resembling a Meckel-like cartilage (white arrow). (J) Overexpression of *Six2* does not affect *Hoxa2* levels. Semi-quantitative RT-PCR on RNA extracted from E10.5 second arches of wild-type and *a2-Six2* (tg) embryos, using specific primers for *Six2*, *Hoxa2* and *GADPH*. g, greater horn; i, incus; l, lesser horn; m, malleus; s, styloid process; st, stapes; t, tympanic ring.

### *Hoxa2* is sufficient to downregulate *Six2* in the head mesenchyme

*Six2* is widely expressed in the head mesenchyme (Oliver et al., 1995). By contrast, *Hoxa2* expression is mainly confined to the second branchial arch (Prince and Lumsden, 1994; Nonchev et al., 1996; Mallo, 1997). To gain insight into how *Hoxa2* regulates *Six2*, we asked whether Hoxa2 repressor activity is restricted to the second arch, or, alternatively, if *Hoxa2* is sufficient to downregulate *Six2* expression in the craniofacial mesenchyme. For this, we took a transgenic approach and ectopically expressed *Hoxa2* under the *Msx2* promoter, able to direct gene expression to the head mesenchyme (Liu et al., 1994; Kanzler et al., 1998).

To detect expression of *Six2* and *Hoxa2* in the same embryo, E10.5 control and *Msx2-Hoxa2* transgenic littermate embryo halves were hybridized with either *Six2* or *Hoxa2* probe. *Six2* mRNA showed an abundant distribution in the maxillary and nasal mesenchyme of wild-type E10.5 embryos, areas that are negative for *Hoxa2* expression (Fig. 4A). Transgenic E10.5 littermates (*Msx2-Hoxa2; n*=4) displayed a markedly reduced expression of *Six2* in the maxillary mesenchyme and to a lesser extent in the periocular mesenchyme, the areas of *Hoxa2* ectopic expression (Fig. 4B). We conclude that *Hoxa2* is sufficient to repress *Six2* in the head mesenchyme in vivo; this observation is particularly interesting because this very same transgenic expression results in reduction or absence of the facial skeleton (Kanzler et al., 1998).

### Identification of a fragment of the *Six2* promoter responsive to Hoxa2

As a transcription factor, Hoxa2 could block the accumulation of *Six2* mRNA in the second arch by directly repressing its transcription. Alternatively, the absence of *Six2* in the second branchial arch could be achieved indirectly, as the result of more upstream events that are in turn regulated by Hoxa2.

As a first step in understanding the role of Hoxa2 in regulating *Six2*, we examined mouse and human genomic *Six2* sequences. The sequence from –900 bp to the putative transcriptional start site (identified as the 5′ end of the *Six2* first exon, GenBank NM_011380) displayed a high conservation between the two species.

To assay whether this promoter region harbors regulatory elements controlling *Six2* transcription, we cloned it upstream of a *lacZ* reporter gene and injected the resulting construct (*900Six2-lacZ*) into fertilized mouse oocytes. Transgenic embryos, collected at E11.5, showed a β-galactosidase (β-gal) staining consistent with the *Six2* expression pattern at various embryo locations, including the branchial area (Fig. 5A) (E.K. and N.B., unpublished). Here, the most proximal 900 bp of the *Six2* promoter directed *lacZ* expression in the proximal part of the first branchial arch (arrowhead) and in mesenchyme proximal to the first branchial arch (arrow), a pattern that faithfully recapitulates endogenous *Six2* expression. The activity of the transgene in the second branchial arch appeared stronger compared with the restricted *Six2* endogenous expression in this area (Fig. 1B). Insertion of the transgene in multiple copies may have reduced the efficiency of Hoxa2 repressor activity; alternatively, other repressor-responsive elements controlling Six2 transcription independently from Hoxa2 may not be contained in our transgene.

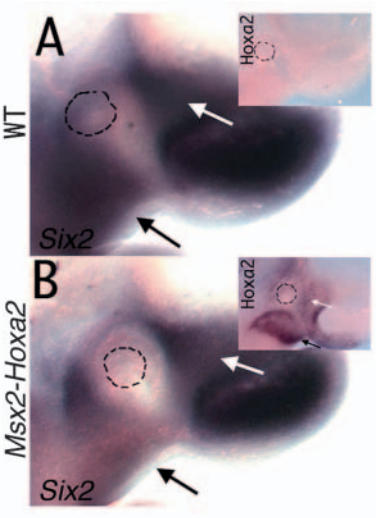


**Fig. 4.** *Hoxa2* is sufficient to repress *Six2* in the facial mesenchyme. (A,B) In-situ hybridization with *Six2* probe, in E10.5 wild-type embryo (A) and in E10.5 *Msx2-Hoxa2* transgenic embryo (B). *Six2* expression is downregulated in the maxilla (black arrow) and periocular mesenchyme (white arrow) of *Msx2-Hoxa2* embryos. The corresponding embryo halves hybridized with the *Hoxa2* probe are shown in the insets.

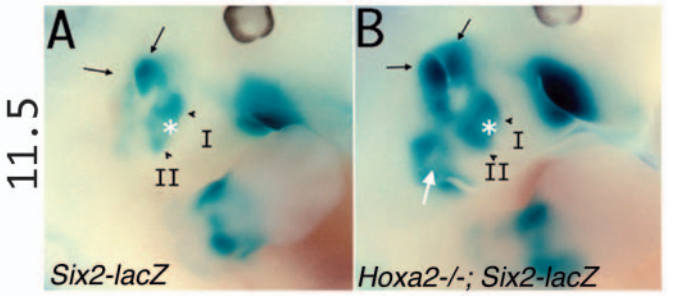


**Fig. 5.** Identification of a promoter fragment responsive to Hoxa2. (A) The most proximal 900 bp of the *Six2* promoter (–893; +37) are sufficient to drive *lacZ* expression in the proximal area of the first branchial arch, similarly to *Six2* endogenous expression. (B) In the absence of Hoxa2, an equivalent staining to the first arch was observed in the second branchial arch (arrowheads) and in the mesenchyme proximal to the branchial arches (arrows), reproducing the *Six2* expression pattern observed in *Hoxa2* mutant embryos (see Fig. 1; white asterisk: first cleft). Higher expression of the transgene was also observed in the caudal area of the second branchial arch, where *Six2* is not differentially regulated (white arrow). I, first branchial arch; II, second branchial arch.

The identified *Six2* promoter region recapitulated the expression of the endogenous gene in the first arch; Hoxa2 blocked *Six2* expression in an equivalent domain in the second branchial arch. To test if this promoter region retains Hoxa2-dependent regulation, we introduced the *900Six2-lacZ* transgene into the *Hoxa2* mutant background. In the absence of Hoxa2, a higher expression of the transgene was observed in the second arch, where β-gal staining became equivalent to that observed in the first arch; the *lacZ*-expressing areas were arranged symmetrically with respect to the first cleft (Fig. 5B, arrowheads). As for endogenous *Six2* signal, *lacZ* expression was also upregulated in the mesenchyme proximal to the branchial arches (black arrows). All together, *lacZ* expression

reproduced the expression pattern of *Six2* in *Hoxa2* mutant embryos. In addition, higher activity of the transgene was observed in the caudal part of the second branchial arch, in an area in which *Six2* is not differentially regulated (white arrow).

These results show that the proximal region of the *Six2* promoter is sufficient to direct *Six2* expression in the branchial area. More importantly, they demonstrate that Hoxa2 controls the *Six2* gene at the transcriptional level and that this control, direct or indirect, is confined to a proximal 900 bp of genomic sequence.

### Hoxa2 directly interacts with *Six2* promoter sequences

Our data suggest the possibility that Hoxa2 may directly repress *Six2* transcription, interacting with the proximal 900 bp of the *Six2* promoter. As noted above, the first kilobase of *Six2* genomic sequences upstream of the transcription start site (TSS; +1) is highly similar between mouse and human. Sequence conservation is extremely high between position –249 and –11, reaching 95% similarity between the two species. To test whether Hoxa2 directly represses *Six2* via binding to the *Six2* promoter, we performed an electrophoretic mobility shift assay (EMSA) using the conserved *Six2* region located immediately upstream of the TATA box (*Bst*EII-*Ssp*I fragment; Fig. 6A) as a probe. Incubation of the probe with in-vitro translated HA-tagged Hoxa2 resulted in the formation of two retarded complexes. These bands represent the interaction of Hoxa2-HA with the probe, as they were supershifted by the addition of the anti-HA antibody. By contrast, incubation of the probe in the presence of unprogrammed reticulocytes did not result in any retarded complex, nor did addition of the antibody have any effect (Fig. 6B). When the probe was fragmented and each half-fragment (probe 1 and probe 2) incubated with Hoxa2-HA, we still observed formation of the characteristic doublet (Fig. 6C,D). A close inspection of the *Bst*EII-*Ssp*I genomic area revealed the presence of two conserved 5′-GAATAAT-3′ motifs, one in each of the two fragmented probes. According to in vitro binding experiments, the Hox consensus sequence contains a TAAT core (Graba et al., 1997); to test whether Hoxa2 recognizes the GAATAAT sequence, we performed competition

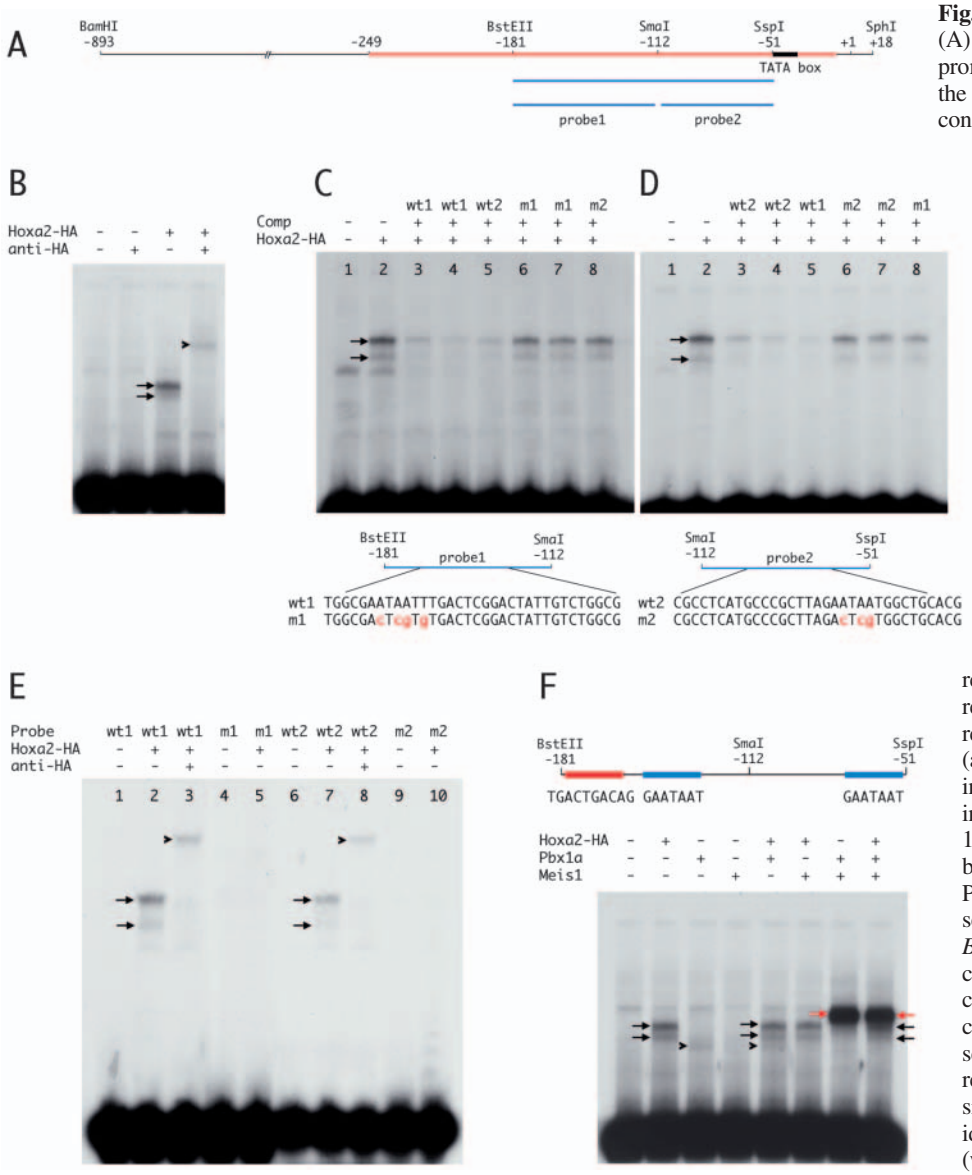


**Fig. 6.** Hoxa2 binds the *Six2* promoter. (A) Schematic representation of the 900*Six2* promoter: the black rectangle corresponds to the TATA box. Sequences showing 95% conservation between mouse and human are underlined in red, probes used in bindshift, in blue. (B) Hoxa2-HA binds to *Bst*EII-*Sma*I probe, and the two retarded complexes (arrows) are supershifted by the anti-HA antibody (arrowhead). (C,D) Hoxa2-HA binds to probes 1 (C) and 2 (D). The addition of cold wild-type double-stranded oligonucleotides (wt1, wt2), but not of mutant oligonucleotides (m1, m2), competes the formation of the complexes (arrows). The sequence of the wild-type and mutant oligonucleotides and their relative position on the promoter are shown; red lowercase letters indicate the introduced nucleotide changes. Cold oligonucleotides were added at 250-fold (3,6) and 500-fold (4,5,7,8,) molar excess. (E) The incubation of Hoxa2-HA with labeled wild-type oligonucleotides results in the formation of the same specific retarded complexes (arrows, 2, 7), recognized by anti-HA antibody (arrowheads, 3, 8). No protein/DNA interaction is observed when Hoxa2-HA is incubated with mutant oligonucleotides (5, 10). (F) Pbx1a and Meis1 cooperate in binding to the proximal *Six2* promoter. Pbx1a, Meis1 and Hoxa2 were incubated, separately or in combination, with the *Bst*EII-*Ssp*I probe. Hoxa2-HA/DNA complex, black arrows; Pbx1a/DNA complex, arrowhead; Pbx1a/Meis1/DNA complex, red arrow. The position and the sequence of the putative Hoxa2 (blue rectangles) and Pbx/Meis (red rectangle) sites are indicated. The Pbx/Meis site was identified using Patch search at Biobase (www.gene-regulation.com).

experiments using wild-type and mutant oligonucleotides. The complex formed in the presence of probe 1 (*Bst*EII-*Sma*I, Fig. 6A) and Hoxa2-HA was competed at two different concentrations of a cold oligonucleotide reproducing the GAATAAT sequence and flanking nucleotides of probe 1. A similar effect was observed upon adding a molar excess of oligonucleotide wt2, which reproduces the GAATAAT motif and flanking nucleotides contained in probe 2 (*Sma*I-*Ssp*I, Fig. 6A). By contrast, the addition of the same molar excess of m1 or m2 oligonucleotides, containing three or four nucleotide substitutions in the GAATAAT, left the complex unaffected (Fig. 6C). Incubation of Hoxa2-HA in the presence of probe 2 resulted in the formation of the same retarded complexes, and these were efficiently competed by wt1 or wt2 oligonucleotides, but not by mutant oligonucleotides (Fig. 6D). The ability of Hoxa2 to recognize the two sites contained in the *Six2* promoter was further confirmed by using the wild-type and mutant oligonucleotides as probes (Fig. 6E). Hoxa2-HA formed slower migrating complexes when incubated with oligonucleotides wt1 and wt2, while no higher complex formation was observed when the mutant oligonucleotides were used as probes in the same assay.

These data show that Hoxa2 interacts with the proximal region of the *Six2* promoter and that this interaction is sequence-specific. Both the identified sites are bound with similar affinity and contain a GAATAAT sequence. Moreover, disruption of their ATAAT core abolishes Hoxa2 binding.

The interaction of Hox proteins with their target promoters often requires co-factors (Mann and Affolter, 1998), and we have indeed detected *Pbx* and *Meis* binding to the *Six2* promoter in close proximity to the Hoxa2 binding sites. Pbx1a alone bound the *Bst*EII/*Ssp*I fragment with very low affinity, while no binding was detectable for Meis1 alone with the probe. When the two proteins were co-translated, an intense retarded band was seen. In contrast to a previous study of Hoxb1 auto-regulatory element (Ferretti et al., 2000), we could not detect the formation of a slower molecular complex by simultaneous incubation of the probe with Hoxa2, Pbx1a and Meis1 (Fig. 6F).

### *Six2* overexpression affects development of the third and more posterior arches

Along with second arch-derived structures, transgenic mice overexpressing *Six2* displayed abnormal growth and morphology of the thyroid and cricoid cartilages and of the hyoid bone, derived from the third and more posterior pharyngeal arches.

The hyoid bone was malformed, curved and fused to the thyroid cartilage, bilaterally or unilaterally (Fig. 7B,C, arrow). Fusion of the greater horn to the lesser horn and to the thyroid cartilage in a structure independent of the main body of the hyoid was often observed (Fig. 7B,C, arrowhead). The thyroid and cricoid cartilages were abnormally thickened, and larger areas of fusion were observed (Fig. 7B,C). Occasionally, the rings of the trachea were abnormally fused to each other and disorganized (Fig. 7C).

*Hoxa3*, *Hoxb3* and *Hoxd3*, alone or in combination, control the formation of mesenchymal derivatives of the neural crest (Chisaka and Capecchi, 1991; Condie and Capecchi, 1993; Condie and Capecchi, 1994; Manley and Capecchi, 1997). Intriguingly, skeletal defects observed in mice carrying mutations in the Hox paralogous group 3, such as the fusion of the greater horn to the thyroid cartilage, fusion of the hyoid bone to the thyroid cartilage, and malformation and fusion of thyroid and cricoid cartilages (Condie and Capecchi, 1994) are reminiscent of the phenotype observed in *a2-Six2* transgenic embryos. This suggests a possible genetic interaction of *Six2* with the Hox genes of paralogous group 3. Indeed, paralogous group 3 mutants and *a2-Six2* transgenic embryos also display common defects in the growth and differentiation of skeletal elements derived from somitic mesoderm (Condie and Capecchi, 1994; Manley and Capecchi, 1997). Fusion of the atlas to the exoccipital bone, a characteristic of the *Hoxd3* mutant phenotype (Condie and Capecchi, 1994; Manley and Capecchi, 1997), was observed in transgenic embryos overexpressing *Six2* (Fig. 7D,E). Similarly to a *Hoxa3; Hoxb3* double mutant (Manley and Capecchi, 1997), the supraoccipital bone displayed only very partial ossification (Fig. 7F,G). These defects are consistent with the high expression of the transgene detected in the anterior somitic mesoderm (Fig. 3D).

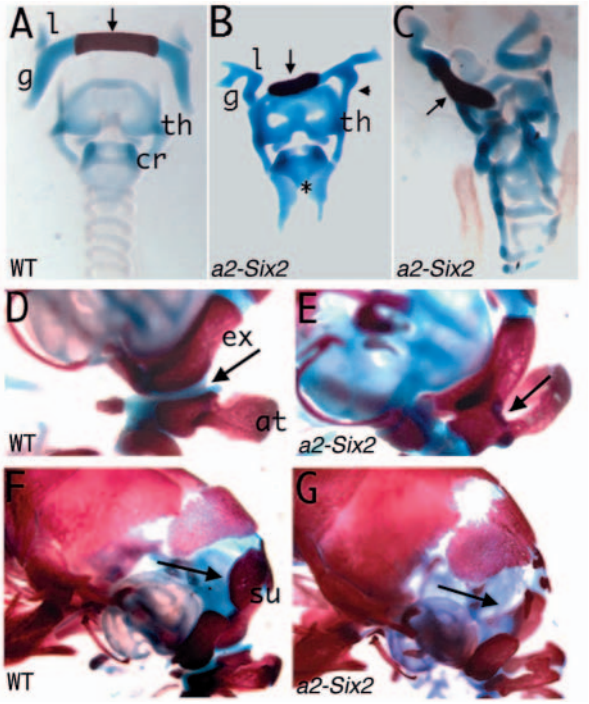


**Fig. 7.** Skeletal phenotype of transgenic embryos. Wild-type (A,D,F) and Six2 transgenic embryos (B,C,E,G) are shown. (A) Hyoid bone (black arrow), greater and lesser horn, and thyroid and cricoid cartilage of wild-type embryos. (B) The hyoid bone (black arrow) is malformed and fused to the thyroid cartilage. The greater horn is fused to the thyroid cartilage (arrowhead) and to the lesser horn. The thyroid and cricoid cartilages are thickened and the cricoid cartilage elongates ventrally (asterisk). (C) The hyoid bone (arrow) and the laringeal cartilages have fused in a disorganized structure. The rings of the trachea are also fused to each other. (D) The exoccipital bone and the atlas are separated by cartilage (arrow). (E) The corresponding area in transgenic embryos (arrow) displays fusion of the atlas to the exoccipital bone. (F) Ossification is almost completed in the supraoccipital bone (arrow). (G) In transgenic embryos the supraoccipital bone shows very reduced ossification (arrow points to the expected position for the supraoccipital bone). at, atlas; cr, cricoid cartilage; ex, exoccipital bone; g, greater horn; l, lesser horn; su, supraoccipital bone; th, thyroid cartilage.

## Discussion

Here we have shown that *Hoxa2* regulates the expression of *Six2* in the second branchial arch. Two main observations suggest that Hoxa2 repression of *Six2* has a functional effect: (1) gain of function of *Six2* in the second arch results in ectopic cartilage formation with characteristics similar to the *Hoxa2* phenotype; (2) Hoxa2 controls the activity of a *Six2* promoter fragment in vivo and binds within this promoter in vitro.

In patterning the embryo, Hox gene activity has been proposed to be transduced by a battery of genes, termed the realizator genes, that directly influence cell processes such as cell adhesion, apoptosis or rate of cell division (Garcia-Bellido, 1975). Like Hoxa2, Six2 is a transcription factor. The next crucial question will therefore be to identify the cellular processes controlled by Six2 in the developing branchial arches.

### *Hoxa2* and second arch patterning

The *Hoxa2* mutation affects skeletal development of the second branchial arch (Gendron-Maguire et al., 1993; Rijli et al., 1993; Barrow and Capecchi, 1999). Second arch skeletal elements are lost and replaced by first arch duplicated elements arranged in a mirror image disposition with respect to their first arch counterparts. How does *Hoxa2* pattern second arch skeleton? Previous reports have shown that *Hoxa2* negatively interferes with the development of the facial skeleton, and in the second arch *Hoxa2* surrounds, but is excluded from, the endochondral ossification centers required to form the hyoid cartilage (Kanzler et al., 1998; Creuzet et al., 2002). The most likely mechanism of action is that Hoxa2 restricts skeletogenesis in the second arch, preventing the formation of first arch duplications, while, at the same time, shaping second arch-specific elements. *Six2* could be one of the genes regulated by *Hoxa2* to restrict skeletogenesis in the second arch. In support of this, *Six2* overexpression in the second branchial arch interferes with the normal patterning of the second arch-derived skeleton; endochondral ossification is increased overall, producing a skeletal phenotype reminiscent of the *Hoxa2* phenotype. Discrepancies in the shape of the skeletal elements between transgenic and mutant embryos indicate that other factors, acting in parallel or in concert with Six2, are required to generate the full *Hoxa2* phenotype.

However, even in the scenario with Six2 as the only factor responsible for the *Hoxa2* phenotype, the few intrinsic differences that characterize the mutant and transgenic second arches could alone account for discrepancies in the final shape of the skeletal elements. First, being driven by a heterologous promoter, the expression pattern of *Six2* in the second arch of transgenic embryos is different from *Six2* endogenous expression in the second arch of the mutant. Second, in contrast to the mutant, a functional Hoxa2 protein is present in the second arch of transgenic embryos.

Final evidence that repression of *Six2* is one of the mechanisms employed by *Hoxa2* in second arch patterning will require analysis of a *Hoxa2*; *Six2* double mutant. Our prediction is that *Six2* inactivation should, at least partially, rescue the *Hoxa2* phenotype. The lack of a *Six2* mutant hampers the accomplishment of this experiment, but the inactivation of *Six1*, the closest homolog to *Six2,* supports our prediction. *Six1* mutant mice display an evident craniofacial phenotype, thereby identifying Six genes as important regulators of neural-crest-derived craniofacial skeleton (Laclef et al., 2003b; Ozaki et al., 2004). We found, both by RT-PCR (see Fig. 2) and by in-situ hybridization (B.E. and N.B., unpublished), that *Six1* is not regulated by *Hoxa2*. However, *Six1* is required for development of part of the skeleton that is affected by the *Hoxa2* mutation (Laclef et al., 2003b; Ozaki et al., 2004), suggesting, together with the high similarity in the encoded proteins and the expression pattern of the *Six1* and *Six2* genes (Oliver et al., 1995), a functional contribution of *Six2* to the *Hoxa2* phenotype.

In the *Six1* mutant, second-arch-derived cartilages fail to form (Laclef et al., 2003b; Ozaki et al., 2004). If *Six2* is also required for second arch skeletal growth, a likely scenario for second arch skeletal patterning is that *Hoxa2* 'tunes' the size of the skeletal elements to be produced in the second arch by regulating the domain of *Six2* expression in this area.

### *Hoxa2* and the formation of the facial skeleton

Evidence accumulated in recent years indicates that Hox genes inhibit development of the facial skeleton in the areas where they are expressed. Gain of function of *Hoxa2*, both in pre-migratory neural crest (Couly, 2002) and in the facial mesenchyme (Kanzler, 1998), prevents the formation of the facial skeleton. In the second branchial arch, its normal domain of expression, *Hoxa2* negatively regulates skeletal development (Kanzler et al., 1998). If the broad effects of *Hoxa2* on skeletal development are attributable to a general mechanism, we should expect the same molecular mediators acting downstream of *Hoxa2* in the facial mesenchyme and in the second branchial arch. An important requisite for such mediators would be a broad expression in the area fated to form head cartilages and bones. We would also expect that gain of function of Hoxa2 would affect the spatial distribution of such a mediator. Indeed, *Six2* displays a widespread expression in the craniofacial mesenchyme (Oliver et al., 1995) and its expression is downregulated following ectopic expression of *Hoxa2*. However, while *Hoxa2* indistinctly inhibits bone and cartilage formation, overexpression of *Six2* in the second branchial arch produces ectopic cartilages but does not affect intramembranous bone growth (the tympanic ring is fairly normal). Does *Six2* specifically promote cartilage formation? The craniofacial defects of the *Six1* mutant are not restricted to cartilage, but affect bones as well (Laclef et al., 2003b; Ozaki et al., 2004). In addition, the domain of *Six2* upregulation around the otic vesicle is spatially associated with the squamous bone duplication observed in the *Hoxa2* mutant. Finally, the analysis of Six mutants indicate that Six genes positively regulate cell proliferation, which explains how these genes control processes as diverse as muscle formation, retina development and skeletal development. On this basis, a likely prediction would be that *Six2* promotes both cartilage and bone development. The lack of effect on bone development in our gain-of-function experiment could be explained by the absence, in the wild-type second arch, of a factor acting in concert with Six2. Alternatively, as the process of intramembranous ossification begins later than chondrogenesis, *Six2* overexpression might occur too early to affect bone formation. We favor the second hypothesis, because *a2-Six2* transgenics show a high level of *Six2* mRNA at E10.5; 1 day later, there are barely detectable differences in *Six2* mRNA levels between transgenics and wild-type embryos (data not shown).

### Repression of *Six2* by Hoxa2

Despite the vast literature on Hox genes, the nature of the genes regulated by Hox proteins in vertebrates is still largely unknown. Solving this riddle is fundamental, if we want to explain how Hox genes control development in the vertebrate embryo.

We have shown that Hoxa2 controls the *Six2* gene at the transcriptional level, as indicated by the identification of a *Six2* promoter fragment regulated by Hoxa2. As a transcription factor, Hoxa2 could directly regulate the *Six2* promoter, and the observed binding of Hoxa2 to this promoter fragment in vitro strongly suggests that *Six2* is a direct target of Hoxa2.

Within the *Six2* promoter, Hoxa2 recognizes two GAATAAT motifs near the transcription start site. The consensus Hoxa2 binding motif has not been previously described, but the sequence recognized by Hoxa2 on the *Six2* promoter meets the requirements for Hox proteins binding to DNA (Graba et al., 1997). The interaction of Hox proteins with their target promoters often requires co-factors such as Pbx (Mann and Affolter, 1998), and we have indeed detected Pbx and Meis binding to the Six2 promoter, in close proximity to the Hoxa2 binding sites. Another hint, albeit indirect, that *Six2* might be a direct target of Hoxa2 is that the absence of Hoxa2 in the second arch specifically affects *Six2* expression, leaving the levels of *Six1*, *Six4* and *Six5* unaffected.

Additional analyses will be required to definitely prove that *Six2* is a direct target of Hoxa2. However, the experimental analysis of *Six2* regulation by Hoxa2 is complicated by the fact that Hoxa2 behaves as a repressor. Acting as a repressor, Hoxa2 might inhibit the basal transcription machinery, counteract the activity of a positively acting transcription factor or, alternatively, interact with proteins that remodel chromatin (Gaston and Jayaraman, 2003). The elucidation of most of these mechanisms will be greatly facilitated by the identification of the proteins acting as activators of *Six2*. Currently, efforts in this direction are proceeding in our laboratory.

### *Six2*: a common molecular target of Hox genes?

Regulation of *Six2* by Hox genes was described in the *Hoxa11; Hoxc11; Hoxd11* mutant, characterized by loss of metanephric kidney induction (Wellik et al., 2002). As in the *Eya1* mutant (Xu et al., 1999), which has a remarkably similar phenotype, in the *Hox11* triple mutants *Six2* expression disappears (Wellik et al., 2002). This effect is opposite to the one observed in the second arch, where *Hoxa2* represses *Six2* expression.

Overexpression of *Six2* under the *Hoxa2* enhancer resulted in profound effects on the development of the skeleton patterned by Hox genes of paralogous group 3; some of the defects observed in Hox paralogous 3 single and compound mutants were mimicked by *Six2* gain of function (consistent with ectopic expression of the transgene in the branchial arches and somitic mesoderm).

All together, these observations raise the possibility that *Six2* expression might be under the control of other Hox genes in the development of the vertebrate embryo. In *Drosophila*, common targets for different Hox proteins have been described, as well as the ability of Hox proteins to behave both as activators and repressors on their target genes (reviewed in Graba et al., 1997). To learn whether this holds true in vertebrates and if *Six2* is a common target of different Hox proteins will require a profile of Six2 expression patterns in different Hox mutants, the entire spectrum of which is currently available.

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