Temporal requirement of \textit{Hoxa2} in cranial neural crest skeletal morphogenesis

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Summary

Little is known about the spatiotemporal requirement of Hox gene patterning activity in vertebrates. In \textit{Hoxa2} mouse mutants, the hyoid skeleton is replaced by a duplicated set of mandibular and middle ear structures. Here, we show that \textit{Hoxa2} is selectively required in cranial neural crest cells (NCCs). Moreover, we used a Cre-ERT2 recombinase system to induce a temporally controlled \textit{Hoxa2} deletion in the mouse. \textit{Hoxa2} inactivation after cranial NCC migration into branchial arches resulted in homeotic transformation of hyoid into mandibular arch skeletal derivatives, reproducing the conventional \textit{Hoxa2} knockout phenotype, and induced rapid changes in \textit{Alx4}, \textit{Bapx1}, \textit{Six2} and \textit{Msx1} expression patterns. Thus, hyoid NCCs retain a remarkable degree of plasticity even after their migration in the arch, and require \textit{Hoxa2} as an integral component of their morphogenetic program. Moreover, subpopulations of postmigratory NCCs required \textit{Hoxa2} at discrete time points to pattern distinct derivatives. This study provides the first temporal inactivation of a vertebrate Hox gene and illustrates Hox requirement during late morphogenetic processes.

Key words: Hox, Cranial neural crest plasticity, Craniofacial development, Mouse, CreERT2, Middle ear, Goniaic bone, Skeletal derivatives, Conditional knockout

Introduction

The morphogenesis of the head region involves the neural crest (NC), a unique cell population that represents one of the innovative traits of the vertebrate lineage. Neural crest cells (NCCs) originate from a dorsal level of the developing neural tube and have the distinctive ability to migrate towards various regions of the embryo, where they differentiate into a variety of cellular types and contribute to the ontogenesis of several organs and structures (reviewed by Gammill and Bronner-Fraser, 2003; Le Douarin and Kalcheim, 1999). In particular, a large part of the head and facial skeleton derives from the NCCs of the cranial region. The cranial NCCs migrating from the diencephalon and anterior mesencephalon contribute to the frontonasal mass and skull bones, whereas NCC populations migrating from posterior mesencephalon and hindbrain contribute to the branchial arches and generate facial, jaw and neck bones (reviewed by Santagati and Rijli, 2003).

The way each subpopulation of cranial NCCs acquires the competence to generate distinctive cartilaginous structures and bones represents a fascinating matter of investigation that has inspired seminal experiments and a fervent discussion over the past few decades. By means of chick-to-quail transplants of pre-migratory NCCs, Noden observed that grafting of presumptive first branchial arch NCCs to replace presumptive second arch NCCs resulted in the mirror duplication of first arch skeletal elements in the place of second arch structures (Noden, 1983). This result suggested that NCCs were intrinsically endowed with the potentiality to form specific structures, or pre-patterned, according to their rostrocaudal origin in the neural tube. However, recent work has shown that the surrounding environment provides instructive signals to NCCs in order to trigger their differentiation process, as well as to achieve the correct shaping, positioning and orientation of the developing skeletal structures (Couly et al., 2002; Crump et al., 2004; Ruhin et al., 2003; Trainor et al., 2002). In turn, the NCCs initiate morphogenesis following an intrinsic, species-specific, patterning program that may involve feedback signalling to epithelia (Eames and Schneider, 2005; Ferguson et al., 2000; Schneider and Helms, 2003; Shigetani et al., 2002; Tucker and Lumsden, 2004). Thus, craniofacial morphogenesis appears to be the result of reciprocal signalling between neural crest mesenchyme and the surrounding environment, where timing is an essential component (reviewed by Le Douarin et al., 2004; Santagati and Rijli, 2003). Although a significant advance has been provided by the identification of molecules influencing cranial NCC patterning ability (reviewed by Helms et al., 2005), little is known about the temporal span during which cranial NCCs can generate an appropriate pattern in response to extrinsic stimuli. Another aspect that is poorly understood is how positional information along the anteroposterior axis of distinct subpopulations of NCCs impinges on the patterning programs to give rise to specific subsets of skeletal elements. These issues are related to the
spatiotemporal plasticity of the NCCs during craniofacial development.

An important molecular distinction of cranial NCCs populations along the anteroposterior axis concerns their patterns of Hox gene expression. The NCCs contributing to skull bones, frontonasal and first arch-derived jaw structures do not express Hox genes (Coulby et al., 1998) (reviewed by Shigetani et al., 2005). By contrast, NCCs contributing to hyoid and throat skeletal elements derived from the second and more posterior branchial arches, respectively, do express various combinations of Hox genes (Hunt et al., 1991). The involvement of Hox genes in providing rostrocaudal patterning information to branchial arch derivatives first became evident with the targeted inactivation of Hoxa2 in the mouse (Gendron-Maguire et al., 1993; Rijli et al., 1993). As a result of Hoxa2 functional depletion, the second arch skeletal elements were homeotically transformed in a duplicated set of first arch-like elements with reverse polarity (Gendron-Maguire et al., 1993; Rijli et al., 1993), strikingly phenocopying the outcome of Noden’s heterotopic grafts in the chick. This result indicated that Hoxa2 acts as a key genetic switch, enabling the patterning of second arch instead of first arch derivatives. Hoxa2 gain-of-function experiments in the first branchial arch of chick, Xenopus and zebrafish embryos yielded a reverse outcome, i.e. second arch-like structures developed in the place of first arch elements, demonstrating a conserved role of Hoxa2 as a selector of second arch patterning information in vertebrates (Grammatopoulos et al., 2000; Hunter and Prince, 2002; Pasqualetti et al., 2000).

Given the unique role of Hoxa2, its mutagenesis in the mouse represents a suitable mammalian genetic model with which to address unsolved questions about the spatiotemporal control of cranial NCC patterning. Here, we set up a conditional mutagenesis system in the mouse, and observed the effects of Hoxa2 deletion in a tissue- and time-dependent manner. Our analysis yielded several novel insights. First, we found that Hoxa2 is selectively required in second arch NCCs. Removal of Hoxa2 in NCCs phenocopied the full knockout transformation of second into first arch morphology. Second, by using a Cre/loxP-based system, we temporally induced Hoxa2 deletion and showed that Hoxa2 function at pre-migratory stages does not provide NCCs with irreversibly information for patterning second arch derivatives. Rather, instruction about shape, size and orientation of second arch skeletal elements is provided after NCC migration. However, the execution of the NCC patterning program is strictly Hoxa2 dependent. In fact, homeotic changes can still be obtained upon Hoxa2 inactivation well after NCCs reached their final destination in the second arch. These data illustrate for the first time that second arch NCCs not only retain a degree of plasticity over a remarkably long period, but also that they require the expression of Hoxa2 as an irreplaceable and integral component of their intrinsic patterning program. Our data also suggest that Hoxa2 may directly regulate the spatial expression of a number of target genes involved in the morphogenetic process. Finally, we found that Hoxa2 function is required at separate time points to pattern distinct second arch derivatives. Altogether, this study provides the first temporal analysis of Hox gene function in a vertebrate embryo and illustrates a Hox requirement during late morphogenetic processes.

Materials and methods

Generation of the CMV-βactin-Cre-ERT2 transgenic line

The CMV-βactin-Cre-ERT2 (Cre-ERT2) transgene was constructed by introducing a 2 kb fragment containing the Cre-ERT2 fusion cassette (Feil et al., 1997) into the EcoRI site of the pCX vector, which bears the CMV immediate-early (CMV-IE) enhancer/chicken β-actin basal promoter driving ubiquitous constitutive expression. A SalI-Sfil 4.6 kb fragment containing the whole transgenic construct from the CMV-IE enhancer to the rabbit β-globin poly A signal was purified and used for pronuclear injection into fertilized oocytes of CD1 mice.

Founder animals were identified by PCR amplification with Cre-specific primers and bred onto a C57BL/6 genetic background. Offspring carrying the Cre-ERT2 transgene were genotyped by PCR. Nine distinct founder transgenic lines were characterized for integration of the transgene by Southern blot hybridization and for ubiquitous expression of the Cre-ERT2 mRNA by in situ hybridization. The functionality of the Cre-ERT2 fusion protein upon tamoxifen induction was ascertained by crossing it with a ROSA 26R (Soriano, 1999) reporter line. Embryos collected as early as 24 hours after treatment carrying both the ROSA 26R reporter allele and the Cre-ERT2 transgene stained ubiquitously upon X-gal exposure (data not shown). Conversely, no staining was observed on control untreated embryos from the same intercross (not shown). Three transgenic lines (F34, F85 and F105a) were selected after this screening. The data presented in this work were primarily obtained using the F34 line, although the F85 line was used for confirmation purposes when needed. No noticeable differences in the outcomes were observed using the two lines.

Mating scheme

The Cre-ERT2 and the Wnt1-Cre (Danielian et al., 1998) transgenic lines were crossed with either the Hoxa2<sup>2GFP</sup> knock-in (Pasqualetti et al., 2002) or the Hoxa2<sup>neo+</sup> conditional alleles (Ren et al., 2002) for the different experimental purposes. To obtain Hoxa2 conditional homozygous mutant mice, a first breeding round was accomplished to generate compound Hoxa2<sup>2GFP/Cre-ERT2</sup> or Hoxa2<sup>2GFP/Cre-ERT2;Wnt1-Cre</sup> mice, respectively. These animals were in turn mated to Hoxa2<sup>2GFP</sup> mice. Homozygous mutant newborns were obtained with an expected Mendelian ratio. All genotypes were determined by PCR.

Tamoxifen treatment

Tamoxifen (Sigma) was dissolved in pre-warmed corn oil (Sigma) to make a 20 mg/ml solution and stored at 4°C. Tamoxifen was administered orally to pregnant females by means of a gavage syringe. Optimal doses for the different developmental stages were experimentally determined as described in the Results section. In particular, the amount of administered tamoxifen per pregnant female (about 35-40 g of body weight) was estimated as follows: 5 mg at 7.0 dpc, 6 mg at 8.0 dpc, 7 mg at 9.5 dpc, 8 mg at 10.0 dpc, 9 mg at 10.5 dpc and 10 mg at 11.0 dpc. To obtain maximal Cre-ERT2 recombination efficiency at later embryonic stages, double (10 mg at 11.5 dpc and 12.0 dpc) and triple (10 mg at 12.5 dpc, 13.0 dpc and 13.5 dpc) successive administrations (chronic treatment) were provided with 12-hour intervals. For precise embryonic stage estimation at the time of treatment, mated females were checked for vaginal plugs every 2 hours. The time of a detected plug was considered as embryonic day 0. Specimens were collected at the desired time after the last treatment.

Skeletal preparation

Neonatal mice were skinned and eviscerated. Skeletons were fixed overnight in 95% ethanol and then stained in Alcian Blue (750 mg/ml in 80 ml of 95% ethanol, 20 ml of glacial acetic acid) for at least 24 hours. Skeletons were cleared in 2% KOH for 8-10 hours, then 1% KOH overnight and then stained in Alizarin Red (100 μg/ml in 1% KOH) overnight. Further clearance was performed in 20%
glycerol/1% KOH. Skeletons were stocked in 50% glycerol/50% ethanol.

**Immunohistochemistry**

Immunostaining for EGFP on cryosections was performed using a polyclonal rabbit anti-GFP antibody (Molecular Probes Europe BV) and a peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Beckman Coulter France SA). Detection was performed with DAB chromogen (DAKO SA).

**In situ hybridisation**

In situ hybridisation on whole-mount embryos and cryosections was performed as previously described (Samad et al., 2004). The following RNA probes were used: Hoxa2 (Ren et al., 2002), Alx4 (Qu et al., 1997), Bapx1 (Nkx3.2) (Lettice et al., 2001), Mx1 (Robert et al., 1989), Sx2 (Oliver et al., 1995).

**Results**

Selective deletion of *Hoxa2* in second arch neural crest cells is sufficient to induce second-to-first arch homeosis

Most of the defects of the *Hoxa2* knockout mice concern structures of NC origin, including the skeletal elements of the second branchial arch and the external ear pinna (Rijli et al., 1993; Gendron-Maguire et al., 1993). Nevertheless, in addition to in NCCs, *Hoxa2* is also expressed in the neural epithelium, where NCCs arise, and in the branchial arch surface ectoderm (Couly et al., 1998; Hunt et al., 1991; Pasqualetti et al., 2000; Prince and Lumsden, 1994). Such tissues are likely to exhibit a cross-talk interaction with the NCCs and to influence their morphogenetic potential. To ascertain whether the *Hoxa2* mutant phenotype is entirely generated by the lack of *Hoxa2* function in the NCCs, or if it is partially contributed by *Hoxa2* loss in neighboring tissues, we conditionally ablated *Hoxa2* in the NCC population. For this purpose, we mated mice homozygous for a previously described *Hoxa2*lox allele (Ren et al., 2002) to a *Wnt1-cre* transgenic line, expressing the Cre recombinase in NCC precursors at the dorsal folds of the neuroepithelium (Danielian et al., 1998). In *Hoxa2*lox/lox;*Wnt1-cre* 10.5 day post coitum (dpc) embryos, *Hoxa2* expression was selectively abolished from the NCCs contributing to the second and more posterior arches, as assessed by whole-mount in situ hybridisation (Fig. 1C) (see also Ren et al., 2002). At birth, *Hoxa2*lox/lox;*Wnt1-cre* mutant mice displayed a similar external phenotype to the conventional *Hoxa2-/-* mutants (Rijli et al., 1993), namely the loss of external ear (Fig. 4C). Also, the analysis of skeletal preparations from *Hoxa2*lox/lox;*Wnt1-cre* mutant pups revealed the same set of malformations as in *Hoxa2-/-* mice. Namely, the second arch-derived stapes, styloid process, and lesser horns of the hyoid bone were absent and replaced by a duplicated set of first arch-like structures with reverse polarity, including a duplicated incus, malleus, tympanic bone, a transformed gonia1 bone, and a partially duplicated Meckel's cartilage (Fig. 1F).

Thus, *Hoxa2* is selectively required in second arch NCCs. *Hoxa2* inactivation in NCCs is sufficient to yield the defects associated with middle and external ear development, while expression of *Hoxa2* in adjacent tissues does not alleviate the overall mutant phenotype.

**Temporally controlled deletion of the *Hoxa2* locus**

In order to carry out a time-course mutagenesis of the *Hoxa2* gene, we generated the transgenic mouse line CMV-β-actin-Cre-ERT2 (*Cre-ERT2*) bearing a tamoxifen (TM)-inducible form of Cre recombinase, *Cre-ERT2* (Feil et al., 1997), driven by the ubiquitous chicken β-actin promoter. Transgenic lines expressing *Cre-ERT2* can be efficiently used to perform time-specific excision of a target gene by a Cre/loxP-based conditional knockout system (reviewed by Metzger et al., 2005). We tested the efficiency of the Cre-ERT2 recombinase in mediating excision at the *Hoxa2* locus by directly monitoring the levels of residual *Hoxa2* mRNA in TM-induced *Hoxa2*lox/lox;*Cre-ERT2* embryos. The doses and conditions of TM treatment (see Materials and methods) were optimized for different developmental time points on the basis of the maximal degree of Cre-ERT2-induced loss of expression of *Hoxa2*, as detected by whole-mount or tissue section in situ hybridisation. Tamoxifen was administered to pregnant females at 9.5, 10.5 and 11.0 dpc (single gavage), as well as at
11.5 or 12.5 dpc (chronic administration; see Materials and methods), and embryos were collected 24 hours after the last TM administration. We detected no or very little residual Hoxa2 expression as a result of Cre-ERT2 induction at any of the selected time points (Fig. 2B,D,F; and data not shown).

In addition, the effectiveness of the Hoxa2 excision was confirmed by means of a complementary Cre-ERT2-induced ‘gain-of-expression’ approach. To this aim, we cross-mated our Cre-ERT2 transgenic line with the Hoxa2 EGFPfloxNeo line, in which the expression of the EGFP reporter, knocked-in at the Hoxa2 locus, is induced only upon Cre-mediated excision of the adjacent floxed Neo cassette (Pasqualetti et al., 2002). Hoxa2EGFPfloxNeo;Cre-ERT2 embryos were collected 24 hours after the last TM administration. The spatial extent of EGFP activation was compared with that of control specimens of similar stages obtained by mating the Hoxa2EGFPfloxNeo knock-in line with a CMV-Cre transgenic line (Dupe et al., 1997). Both constitutive and induced Cre activities gave rise to similar EGFP immunostaining patterns throughout the Hoxa2 expression domain. In particular, EGFP staining in the second arch from both genotypes was indistinguishable at any of the probed stages (Fig. 2G,H; and data not shown).

Distinct temporal requirements of Hoxa2 in the patterning of second arch structures

In the mouse, Hoxa2 is expressed in second arch NCCs from the onset of their migration (Maconochie et al., 1999) through the stage of prechondrogenic condensation (Kanzler et al., 1998). When chondrogenesis begins, Hoxa2 is downregulated in the cells that undergo differentiation, whereas it remains expressed in the NC-derived surrounding mesenchyme (Kanzler et al., 1998) until at least 14.5 dpc (Fig. 2E), a stage at which chondrogenesis is well underway.

To address the temporal requirement of Hoxa2 in NC patterning, pregnant females were given tamoxifen at different doses and gestational stages following the scheme described above (see also Materials and methods), and Hoxa2flox/flox;Cre-ERT2 fetuses or newborns were collected and analysed for mutant phenotypes. We started by inducing Hoxa2 deletion after TM treatment at 7.0 dpc. TM induces Cre-ERT2 translocation to the nucleus within 6 hours, it reaches its maximal accumulation at about 24 hours and is still present after about 36 hours (Hayashi and McMahon, 2002; Zervas et al., 2004). Considering that skeletogenic NC migration in the mouse begins at around 8.25 dpc (Serbedzija et al., 1992), TM treatment at 7.0 dpc was expected to induce efficient Hoxa2 inactivation before the onset of NCC migration, and therefore to reproduce the phenotypes of conventional and conditional Wnt1-Cre-induced Hoxa2 knockouts. As anticipated, the TM-induced Hoxa2flox/flox;Cre-ERT2 mutant newborns died perinatally, and displayed mirror image duplication of first arch-like structures and absence of external ear pinna (Fig. 3C; and data not shown). This outcome confirmed that the TM-inducible targeting system could be efficiently employed to induce a full Hoxa2 knockout phenotype. Conversely, no morphological abnormalities were detected in Hoxa2flox/flox;Cre-ERT2 newborn mice that did not undergo TM treatment (Fig. 3B).

Next, we tested the effect of Hoxa2 inactivation at NC post-migratory stages. We started with TM treatment at 9.5 dpc. By this stage, skeletogenic NCCs have already completed their migration (Serbedzija et al., 1992) and filled the branchial arches. Notably, deletion of Hoxa2 at this stage also yielded a

**Fig. 2.** Tamoxifen-induced deletion of the Hoxa2 locus. (A-F) In-situ hybridization on whole mounts (A,B) and frontal cryosections (C-F) of mouse embryos using an antisense Hoxa2 probe. Wild-type embryos at 10.5 (A), 13.5 (C) and 14.5 (E) dpc show strong Hoxa2 expression in the second (BA2) and posterior branchial arches (A), as well as in the neural tube (NT) and middle ear regions (C,E), respectively. Significant loss of Hoxa2 transcripts is observed in 10.5 dpc Hoxa2flox/flox;Cre-ERT2 embryos (i.e. carrying two alleles to be excised) after tamoxifen (TM) treatment at 9.5 dpc (B), as well as in 13.5 (D) and 14.5 (F) dpc Hoxa2flox/flox;Cre-ERT2 embryos (i.e. carrying only one allele to be excised) after TM treatment at 11.5 and 12.5 dpc, respectively. (G,H) Immunostaining on frontal cryosections of Hoxa2EGFPfloxNeo;CMV-Cre (G) and Hoxa2EGFPfloxNeo;Cre-ERT2 (H) 13.5 dpc mouse embryos using an anti-GFP antibody. Note that the distribution of TM-induced GFP expression in H is comparatively similar to the constitutive GFP expression in G. OC, otic capsule; S, stapes.
lack of external ear pinna and homeotic replacement of second with first arch-like structures (Fig. 3E,J). Interestingly, the only distinct feature in the middle ear skeleton of ‘post-migratory’ Hoxa2\(^{\text{del/flox}}\);Cre-ERT2 mutants was a rather normal looking gonial bone (Fig. 3E). In the conventional and ‘pre-migratory’ Hoxa2 mutants, this membranous bone appears, on the contrary, transformed, as it is abnormally enlarged and stretches across, bridging over the two halves of the mirror duplication (Rijli et al., 1993) (Fig. 1E,F, Fig. 3A). Notably, deletion of Hoxa2 by TM treatment at 8.0 dpc resulted in a variable gonial bone phenotype, ranging from a malformed gonial bone to a complete transformation of the element (Fig. 3D; data not shown). These results indirectly identify a time window for gonial bone specification and indicate that the first arch NCCs contributing to this membranous element may be irreversibly committed at an early migratory stage, before the NCCs give rise to the other elements of the middle ear region.

We further investigated the effect of Hoxa2 inactivation at later developmental stages. Remarkably, conditional Hoxa2 deletion induced by TM treatment after NCC migration at 10.5 and even at 11.0 dpc still resulted in the duplication of middle ear elements, with the exception of the gonial bone, and in a lack of the lesser horns of the hyoid bone (Fig. 3F,G,K), similar to the outcome of the TM treatment at 9.5 dpc. By contrast, in embryos treated at 11.5 dpc, duplicates of the tympanic and squamosal bones were no longer observed, whereas the second arch cartilaginous derivatives were still affected (Fig. 3H; and data not shown). Specifically, the lesser horns of the hyoid bone were missing (Fig. 3L) while, interestingly, the stapes and the styloid process were malformed and associated with ectopic cartilage (arrows in Fig. 3H), perhaps representing an intermediary state towards the generation of duplicated first arch-like middle ear elements. Finally, in newborns treated at 12.5 dpc, the second arch skeletal elements were only mildly affected (data not shown). The analysis at 11.5 and 12.5 dpc was also carried out on mice bearing a fully deleted and a floxed allele (Hoxa2\(^{\text{del/flox}}\);Cre-ERT2), so that only one allele, instead of two, was to be excised. The results were comparable to those obtained with Hoxa2\(^{\text{del/flox}}\);Cre-ERT2 fetuses (data not shown). Moreover, analysis by in situ hybridisation in late-treated embryos confirmed an essentially complete loss of Hoxa2 expression (Fig. 2D,F). Thus, the obtained phenotypes did not appear to be due to partial Cre-mediated recombination; rather, they unveiled distinct temporal requirements for Hoxa2 in patterning individual skeletal elements and subsets of NCCs populations of the second arch.

The external ear was systematically affected in all TM-induced Hoxa2 mutants. In particular, Hoxa2 mutagenesis induced by TM treatment up to 11.5 dpc always resulted in the loss of the pinna (Fig. 4D,E). In addition, mutant pups obtained by chronic tamoxifen treatments between 12.5 and 13.5 dpc displayed a smaller external ear (Fig. 4F). This indicated a late role of Hoxa2 in the morphogenesis of the external ear pinna, temporally distinct from its involvement in middle ear patterning.

Altogether, these results have revealed an unprecedented plasticity of second arch NCCs, even after they completed their migration. They have also shown that the execution of their morphogenetic program is absolutely dependent on Hoxa2 function at post-migratory stages. Moreover, our data indicate that Hoxa2 exerts temporally distinct functions on specific subpopulations of NCCs within the second arch, each giving rise to individual skeletal elements or external ear structures.

**Hoxa2 inactivation at post-migratory stages results in rapid molecular changes in downstream target genes**

The finding that Hoxa2 acts at late developmental stages, in
concomitance with the onset of the morphogenetic process, prompted us to investigate whether Hoxa2 may be an integral component of the NCC molecular patterning program. In particular, we asked whether Hoxa2 could be directly involved in the generation of the second arch-specific molecular pattern in post-migratory NCCs by regulating the spatial expression of genes involved in branchial arch patterning.

We searched for genes displaying asymmetrical expression patterns in the first and second arch NCC mesenchyme at around the time when morphogenesis is beginning. We first tested whether their expression patterns could be selectively altered in the conventional Hoxa2–/– mutant. Interestingly, genes exclusively or predominantly expressed in the first arch, such as the homeobox genes Alx4, Bapx1 and Six2, were found to be ectopically expressed in the second arch of Hoxa2–/– 10.5 dpc embryos (Fig. 5B,E,K). Conversely, Msx1, which has a broader expression pattern in the hyoid than in the mandibular arch, was downregulated to a first arch-like pattern in the second arch of Hoxa2–/– embryos (Fig. 5H).

Next, we asked whether the expression pattern changes were the consequence of post-migratory Hoxa2 lack of function. We, therefore, induced the Hoxa2 mutation in 9.5 dpc embryos, collected them 24 hours later, and performed whole-mount in situ hybridisation for the selected genes. Postmigratory Hoxa2 inactivation was sufficient to induce similar expression pattern changes of Alx4, Bapx1, Six2 and Msx1 to those detected in conventional Hoxa2 knockout embryos (Fig. 5C,F,I,L), providing molecular support to the morphological transformation of skeletal structures observed in newborns.
Temporal inactivation of *Hoxa2* in the mouse

Temporal inactivation of Hoxa2 in the mouse reveals plasticity of second arch neural crest cells even after their migration

We applied an inducible Cre-ERT2-based approach to achieve time-dependent inactivation of a conditional allele of *Hoxa2*. By this means, we were able to carry out for the first time a functional analysis of the temporal requirement of a Hox factor in a vertebrate embryo. Hox genes are known to play a fundamental role in providing segmental identity at early developmental stages in the vertebrate embryo (Krumlauf, 1994), but little information is available about their potential involvement in late aspects of morphogenetic processes. The importance of this type of study has been recently addressed in a work reporting the conditional inactivation of *Hoxb1* in neurogenic NCCs (Arendt et al., 2004). The authors performed their conditional mutagenesis by employing pre- and post-migratory NCC Cre transgenic lines under the control of two distinct promoters from the *Wnt1* and *Ap2* genes, respectively. However, in this type of approach a precise stage-specific dissection of the role of the gene was precluded. On the one hand, the Cre-ERT2-based recombination approach is an efficient and reliable system for temporally controlled, inducible Cre activity both in adult mouse tissues and developing embryos (Ahn and Joyner, 2004; Danielian et al., 1998; Feil et al., 1997; Hayashi and McMahon, 2002; Imai et al., 2001; Kimmel et al., 2000; Metzger et al., 2005; Sgaier et al., 2005). On the other hand, the use of Cre-ERT2 recombinase regulated by a ubiquitous promoter does not consent, on its own, for a tissue-restricted analysis. In this respect, the finding that the conditional inactivation of *Hoxa2* with a *Wnt1-Cre* line resulted in a full knockout phenotype strongly indicates that the abnormalities observed in our time-dependent mutagenesis are mainly due to the disruption of *Hoxa2* activity in the NCC population.

In the mouse, *Hoxa2* expression in second arch NCCs is present from the time of their emergence from the neural folds, at around 8.25 dpc (Maconochie et al., 1999; Nonchev et al., 1996). We show that such an early expression does not provide NCCs with irreversible patterning information. In fact, inactivation of the *Hoxa2* deletion at post-migratory stages up to at least 11.0 dpc is still sufficient to generate morphological changes substantially similar to those obtained in embryos in which *Hoxa2* is knocked out from NCC pre-migratory stages. Thus, mouse *Hoxa2* is mainly required after the process of NCC migration, in keeping with the maintenance of its expression through at least 14.5 dpc (Fig. 2) (see also Kanzler et al., 1998), a stage at which cartilage differentiation and morphogenesis are underway. This finding also supports the conclusions from the complementary gain-of-function experiments performed on *Xenopus* embryos, in which it was shown that ectopic overexpression of *Hoxa2* mRNA in the Hox-negative first branchial arch could still cause a homeotic first-to-second arch transformation, even at stages subsequent to NCC migration (Pasqualetti et al., 2000).
Most interestingly, the current work reveals for the first time that second arch NCCs maintain plasticity over a remarkably long period, until they undergo skeletal differentiation in the branchial arch, and that they do so irrespective of any previous expression of Hoxa2. As we demonstrate that Hoxa2 is required after NCC migration, our results strongly suggest that Hoxa2 expression in NCCs is necessary for the molecular interpretation of the information provided by the surrounding environment to yield a second arch-specific morphogenetic program. This model integrates the concept of NCC plasticity with the existence of a NC-specific inherent genetic program that responds to extrinsic stimuli taking into account the developmental history and positional origin of distinct NCC subpopulations.

**Distinct temporal and qualitative requirements of Hoxa2 for the morphogenesis of second arch structures**

Vertebrate Hox genes are known to confer anteroposterior positional values to developing tissues, but little is known about how their function is implemented (Knop et al., 2004; Samad et al., 2004; Stadler et al., 2001; Wellik et al., 2002). We show that this function can be accomplished by a localized morphogenetic activity, which, in the case of Hoxa2, is integral to the control of the shape, size and location of the skeletal elements of the second branchial arch.

An important aspect of our study is that we unveiled a differential temporal effect of Hoxa2 inactivation on cartilage and membranous bone development. Indeed, conventional knockout and gain-of-function studies suggested that Hoxa2 is involved in patterning cartilaginous elements (Grammatopoulos et al., 2000; Hunter and Prince, 2002; Pasqualetti et al., 2000; Rijli et al., 1993), while at the same time mediating a general inhibitory activity on membranous bone formation (Creuzet et al., 2002; Kanzler et al., 1998). We now show that these two roles can be temporally dissected. In particular, intramembranous bone formation can be ectopically induced in the second arch by Hoxa2 inactivation up to 11.0 dpc, although not beyond this stage, whereas it is still possible to affect cartilage patterning by TM treatment until at least 11.5 dpc (Fig. 3). This observation indicates that intramembranous bone NC-derived precursors may be specified earlier than cartilage precursors. This is in keeping with the timing of appearance of the early osteoblast differentiation marker Cbfa1 (Runx2), whose expression in the maxillary and mandibular components of the first branchial arch can be detected as early as 11.5 dpc (Otto et al., 1997). In the Hoxa2+/− mutant, Cbfa1 is ectopically expressed in the second branchial arch, implying an inhibitory role of Hoxa2 on the activation of the osteogenic marker (Kanzler et al., 1998). Thus, the fact that ectopic membranous osteogenesis can be triggered in the second arch by TM-treatment not later than 11.0 dpc (Fig. 3G) strongly suggests that commitment of NCCs to an osteogenic fate occurs close to or at the time when Cbfa1 expression is induced, that is around 11.5 dpc, even though the first osteogenic differentiation markers will not appear before 13.0 dpc (Bialek et al., 2004). Beyond 11.5 dpc, second arch NCC precursors devoid of Hoxa2 expression are apparently not able to engage into an osteogenic program (Fig. 3H), either because the inducing signal is not available or possibly because the NCCs have lost their responsiveness.

The inhibition of gonial bone formation represents the sole indication of an early function for Hoxa2, as TM-treatment from 9.5 dpc on did not result in gonial bone transformation (Fig. 3E). It is not clear how the NCCs that will originate the gonial condensation are specified during their migratory phase or entry in the arch. In fact Bapx1, a gene whose expression in the mouse is associated with incudo-mallear articulation and gonial bone development (Tucker et al., 2004), is not detected in the arches until 10.5 dpc, and it is still ectopically activated in the second arch subsequent to post-migratory Hoxa2 inactivation (Fig. 5; see below). It is therefore conceivable that Bapx1 is necessary but not sufficient to promote gonial bone development, and that it requires some other early factor, whose expression can be irreversibly inhibited by Hoxa2 in the presumptive second arch NCCs during or soon after their migration.

Finally, we have shown that Hoxa2 inactivation up to 11.5 dpc can still result in a loss of the pinna, similar to in conventional Hoxa2+/− mice, although homeosis of middle ear elements is no longer observed. Moreover, external ear development can still be affected by Hoxa2 inactivation, even at later stages, resulting in a hypomorphic pinna, whereas middle ear ossicles are normally generated (Fig. 4; and data not shown). Thus, Hoxa2 might be involved in the morphogenesis of the second arch ectomesenchyme, contributing to the pinna through advanced developmental stages, later than its requirement for middle ear patterning. Future studies will be required to assess the molecular pathways regulated by Hoxa2 in external ear morphogenesis.

**Hoxa2 is a positive and negative regulator of gene expression in second arch mesenchyme**

The gene expression analysis conducted in this work has provided additional insights on the molecular program regulated by Hoxa2 in the second arch. Previous studies reported that first arch specific genes can be ectopically activated in the second arch territory of Hoxa2 mutant embryos (Bobola et al., 2003; Kutejova et al., 2005). Thus, it has been proposed that Hoxa2 has a mainly repressive role in the second arch NCCs, and that in some cases it is directly implicated in transcriptional inhibition, as with respect to Six2 (Kutejova et al., 2005). We found here that additional first arch-specific homeobox containing genes, such as Alx4 and Bapx1, are similarly repressed by Hoxa2. However, the expression of Mxl1, which is normally higher and displays a broader distribution in the hyoid than in the mandibular arch at 10.5 dpc, decreases and acquires a first arch-like pattern in the absence of Hoxa2 (Fig. 5). Thus, Hoxa2 can regulate gene expression both positively and negatively, possibly by interacting with specific cofactors, in order to constitute a second arch specific pattern.

It is interesting to note that the asymmetrical distribution between the mandibular and hyoid arches of these gene transcripts is not evident in the NC mesenchyme of 9.5 dpc embryos (data not shown), despite Hoxa2 expression in the second arch. This is a further indication that Hoxa2 mainly plays a later role, noticeable from 10.5 dpc onwards. Interestingly, even at 10.5 dpc, when the arch specific expression pattern has already been established, Hoxa2 inactivation results in homeotic transformation of skeletal elements (Fig. 5). At the molecular level, induction of Hoxa2...
deletion by TM treatment of 9.5 or even 10.5 dpc embryos equally results in a transformation of the second arch gene expression pattern to a first arch-like pattern (Fig. 5; and data not shown). This finding provides strong molecular support to the evidence of long-lasting plasticity of second arch NCCs, as discussed above. In fact, at least up to 10.5-11 dpc, NCCs are still able to undergo rapid molecular changes and switch their developmental course. In this respect, it is also noteworthy that the switch in gene expression upon Hoxa2 inactivation can occur as rapidly as within 12 hours. Considering that tamoxifen takes approximately 6 hours from the time of administration to start accumulating in the nucleus, the time needed to induce a response on the NCC patterning is likely to directly regulate some of those molecular targets. Accordingly, Six2 has recently been proposed to be a direct target of Hoxa2 (Kutejova et al., 2005). However, ectopic expression of Six2 in the second arch NCCs was not sufficient to reproduce the Hoxa2 mutant phenotype (Kutejova et al., 2005), suggesting a requirement for additional players. Indeed, our results suggest that a number of genes involved in patterning NCC derivatives may be under the direct transcriptional control of Hoxa2 to generate a second arch-specific pattern. In this respect, the employment of our inducible system to compare second arch transcripts at different stages between wild type and mutant should allow the identification of the Hoxa2 target genes responsible for the development of distinct structures.

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References


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