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Expressing Hoxa2 across the entire endochondral skeleton alters the shape of the skeletal template in a spatially restricted fashion

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ABSTRACT

Hox genes control morphogenesis along the antero-posterior axis. The skeleton of vertebrates offers an exemplar readout of their activity: Hox genes control the morphology of the skeleton by defining type of vertebrae, and structure of the limbs. The head skeleton of vertebrates is formed by cranial neural crest (CNC), and mainly by a Hox-free domain of the CNC. Ectopic expression of anterior Hox genes in the CNC prevents the formation of the facial skeleton. These inhibitory effects on skeletogenesis are at odds with the recognized function of Hox genes in patterning the developing skeleton. To clarify these controversial effects, we overexpressed *Hoxa2* across the entire developing endochondral skeleton in mouse. This gave rise to strong and spatially restricted effects: the most noticeable abnormalities were detected in the cranial base and consisted in a failure of bones to form or in a transformed morphology of bones. The rest of the skeleton exhibited milder defects, which never consisted in the absence or the transformation of any skeletal components. Analyses at early stages of endochondral bone development showed disorganized cell condensations in the cranial base of *Col2a1-Hoxa2* transgenic embryos. We show that the distribution of *Hoxa2*-positive cells in *Col2a1-Hoxa2* embryos does not match the wild-type developing cartilages. The *Hoxa2*-positive cells detected in atypical, non-chondrogenic location in the cranial base, remain as chondrocytes and lay down cartilage, indicating that Hoxa2 does not alter the fate of chondrocytes, but interferes with their spatial distribution. We propose that the ability of Hoxa2 to change the spatial distribution of cells accounts for the different phenotypes observed in *Col2a1-Hoxa2* embryos; it also provides an explanation for the apparent inconsistency between the inhibitory effects of Hoxa2 on skeletal development, and the ability of Hox genes to establish the morphology of the vertebrate skeleton.

1. Introduction

Hox genes control morphogenesis along the antero-posterior[1] (AP) axis in all animals with bilateral symmetry (Carroll, 1995). The phenotype of Hox mutants is often a spectacular one, characterized by the transformation of a body part into a different one. This phenotype is easier to observe in *Drosophila* than in vertebrates due to the increasing redundancy of Hox genes in higher organisms. However the generation of mice carrying mutations in multiple Hox genes (Wellik and Capecchi, 2003) has confirmed a conserved role for Hox proteins, which is to lay down positional information along the AP axis; this information is then translated by the cells into the appropriate type of structure to be built in that specific body position. As transcription factors Hox proteins are expected to do so by activating and repressing target genes; the nature and function of Hox downstream targets remain elusive in vertebrates (Svingen and Tonissen, 2006; Pearson et al., 2005). One of the best studied readout of Hox genes activity is the vertebrate skeleton. Hox genes control the formation of the skeleton by defining type of vertebrae, and structure of the limbs (Wellik, 2007). Recent work suggests that the patterning activity of Hox proteins is already established in the presomitic mesoderm, well before somites occupy their final position along the embryonic AP axis (Carapuco et al., 2005).

With respect to the axial and appendicular skeleton, the head skeleton of vertebrates has a different origin, as it is largely formed by neural crest. These cells can give rise to many derivatives and only the cranial portion of the neural crest (CNC), as opposed to the trunk neural crest, forms skeleton. CNC cells can differentiate into cartilage and also ossify directly to form bones (Le Douarin and Kalcheim, 1999). Most of the skull

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[1] AP, antero-posterior; CNC, cranial neural crest; ECM, extracellular matrix; HE, hematoxylin eosin; ISH, in situ hybridization; RA, retinoic acid.

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bones are formed by direct or intramembranous ossification, a process that in the axial skeleton only gives rise to the clavicles. The majority of the head skeleton derives from the anterior portion of the CNC; this cell population does not express any Hox genes. *Hoxa2* and *Hoxb2* are the most anterior-expressed Hox genes in the CNC (Prince and Lumsden, 1994; Nonchev et al., 1996; Mallo, 1997; Barrow and Capecchi, 1999). Hoxa2 controls second branchial arch identity: in its absence the skeleton of the second arch (CNC-derived) is transformed into a mirror image duplication of the more anterior first arch skeleton (Barrow and Capecchi, 1999; Gendron-Maguire et al., 1993; Rijli et al., 1993). Forcing the expression of *Hoxa2* in the CNC prevents the formation of the facial skeleton (Couly et al., 2002; Creuzet et al., 2002; Kanzler et al., 1998). This effect, which has been observed in chick and in mouse, by targeting *Hoxa2* expression to the pre-migratory and post-migratory CNC, respectively, is apparently controversial with the recognized role of Hox genes in establishing the morphology of the developing skeleton. It is also unlike the homeotic transformations observed upon gain of function of Hox genes in the axial skeleton (Kessel et al., 1990; Jegalian and De Robertis, 1992; Charite et al., 1994).

Although the facial skeleton has a different embryonic origin with respect to the rest of the skeleton, once CNC cells differentiate into chondrocytes the formation of endochondral bone proceeds via the same general mechanism in the head as in the axial and appendicular skeleton. The failure of the facial skeleton to form when *Hoxa2* is ectopically expressed in the CNC could be due to the interference with a basic, general mechanism of skeletal development, or could specifically target development and/or differentiation of the CNC. In the first case Hoxa2 is expected to generally interfere with the formation of the skeleton; in the second case, its effects would be restricted to the CNC-derived skeleton, i.e. only the head. To distinguish between these two possibilities, we have ectopically expressed *Hoxa2* in all the cells of the embryo fated to become chondrocytes. Although the observed effect was not homogenous, it was not restricted to the CNC-derived skeleton.

2. Material and methods

2.1. Molecular and phenotypic analyses

Tissue sections were analyzed by hematoxylin eosin (HE) according to standard procedure, Alcian blue staining (Pearse, 1968) and in situ hybridization (ISH) (Kanzler et al., 1998) using *Hoxa2* probe (Mallo and Brandlin, 1997). For whole mount analyses, E18.5 embryos were eviscerated, skinned and fixed. Following fixation, cartilages were stained with Alcian blue and bones with alizarin red (Mallo and Brandlin, 1997).

2.2. Mutant and transgenic animals and embryos

The *Col2a1-Hoxa2* transgene contains *Hoxa2* cDNA (Kanzler et al., 1998), provided with the SV40 polyadenylation signal and cloned downstream *Col2a1* promoter/enhancer (Zhou et al., 1995). Transgenic embryos were generated as described (Kanzler et al., 1998) by pronuclear injection of the *Col2a1-Hoxa2* transgene. No transgenic lines were established; all the transgenic embryos analyzed were generated by independent pronuclear injections.

3. Results

3.1. Non-homogenous effects of Hoxa2 on the developing endochondral skeleton

The majority of the vertebrate skeleton is built from a cartilage template. Cells assemble at high density and form mesenchymal condensations; thereafter cells in the condensations differentiate into chondrocytes and lay down cartilage-specific extracellular matrix (ECM). To target the expression of *Hoxa2* to all cells fated to produce cartilage in the developing embryo, we placed the *Hoxa2* cDNA under the control of the *Col2a1* enhancer, specifically active in early chondrocytes (Zhou et al., 1995). Embryos generated by pronuclear injection of the resulting *Col2a1-Hoxa2* transgene were collected at E18.5. Skeletal preparations of wild-type and transgenic embryos, stained with Alcian blue and Alizarin red to visualize cartilage and bone, respectively, showed an abnormal skeletal phenotype in the majority of transgenic embryos (12 out of a total of 15 transgenic embryos, Table 1). The supraoccipital and exoccipital bones in the occipital region of the skull (Fig. 1A, B), and the basioccipital and basisphenoid bones in the posterior cranial base were absent (Fig. 1D) (3 out of the 12 affected transgenic embryos); the otic capsule was also reduced in those embryos. In 4 out of the 12 affected transgenic embryos, the morphology of the cranial base was altered, and the basioccipital and basisphenoid bones displayed a totally different shape (Fig. 1E; we define this radical change in morphology as a "transformation" because the affected structures do not resemble their wild-type counterparts any longer). These strong defects in the head skeleton were often accompanied by abnormalities in the axial and appendicular skeleton; we observed a shorter skeleton, delayed ossification and bell-shaped rib cage in 4 out of the 7 embryos displaying an abnormal head skeleton (Fig. 1F, G). The remaining affected embryos (5 out of the 12 affected transgenic embryos)

Table 1
Summary of skeletal structures affected in E18.5 Col2-Hoxa2 and their embryonic origin.

Structures	Number of transgenics displaying affected structure	Skeletal area	Embryonic origin
Nasal cartilages	0	Head/face	CNC
Presphenoid	4	Head/cranial base	CNC
Basisphenoid	12	Head/cranial base	CNC/paraxial mesoderm
Basioccipital	12	Head/cranial base	paraxial mesoderm
Exoccipital	5	Head/occipital	paraxial mesoderm
Supraoccipital	3	Head/occipital	paraxial mesoderm
Otic capsule	3	Head/occipital	CNC/paraxial mesoderm
Middle ear	0	Head/occipital	CNC
Meckel	3	Head/face	CNC
Hyoid bone	0	Head/neck	CNC
Limbs	3	Appendicular	Lateral mesoderm
Vertebrae	4	Axial	Paraxial mesoderm
Ribs	3	Axial	Paraxial mesoderm