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Agenesis of the Scapula in *Emx2* Homozygous Mutants

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The shoulder and pelvic girdles represent the proximal bones of the appendicular skeleton that connect the anterior and posterior limbs to the body trunk. Although the limb is a well-known model in developmental biology, the genetic mechanisms controlling the development of the more proximal elements of the appendicular skeleton are still unknown. The knock-out of *Pax1* has shown that this gene is involved in patterning the acromion, while the expression pattern candidates *Hoxc6* as a gene involved in scapula development. Surprisingly, we have found that scapula and ilium do not develop in *Emx2* knock-out mice. In the homozygous mutants, developmental abnormalities of the brain cortex, the most anterior structure of the primary axis of the body, are associated with important defects of the girdles, the more proximal elements of the secondary axis. These abnormalities suggest that the molecular mechanisms patterning the more proximal elements of the limb axis are different from those patterning the rest of appendicular skeleton. While *Hox* genes specify the different segments of the more distal part of the appendicular skeleton forming the limb, *Emx2* is concerned with the more proximal elements constituting the girdles. **© 2001 Academic Press**

Key Words: **Emx2; knock-out; scapula; ilium; shoulder girdle; pelvic girdle; appendicular skeleton; limb development.**

INTRODUCTION

Limb development has been extensively investigated by developmental biologists, and recent progress in molecular embryology has provided considerable insight into the genetic control of limb formation and the molecular mechanisms and interactions that pattern the diverse components of the appendicular skeleton. However, little is known about the genes that are specifically involved in the development of the more proximal elements of the appendicular skeleton forming the shoulder and pelvic girdles.

Among the genes that pattern the skeletal elements of the limb, *Hox* genes play a pivotal role. Vertebrate *Hox* genes have shown to be crucial in patterning the primary and secondary axes of the developing embryo. Targeted mutations in mice or analysis of the naturally occurring mutants have made possible a detailed functional investigation of these genes. In the primary axis, *Hox* genes are involved in patterning regional diversity in the branchial region of the head and in the paraxial mesoderm (Kessel and Gruss, 1991; Krumlauf, 1994; McGinnis and Krumlauf, 1992). In the limb, *HoxA* and *HoxD* complexes are involved in the specification and patterning of the stylopodium or upper limb, of the zeugopodium or lower limb, and of the autopodium that comprises the digits (Duboule, 1992; Rijli and Chambon, 1997; Zakany and Duboule, 1999). *Hoxa9/Hoxd9* double mutants show defects at the level of the stylopodium (Fromental Ramain *et al.*, 1996); *Hoxa10/Hoxd11* double mutants have forelimb defects with truncations and deformations at the level of the zeugopodium (Favier *et al.*, 1996); *Hoxa11/Hoxd11* double mutants show a very severe phenotype displaying the almost entire absence of radio and ulna (Davis *et al.*, 1995); *Hoxd12* and *Hoxd13* mutants show digit phenotype (Davis and Capecchi, 1996; Dolle *et al.*, 1993; Zakany and Duboule, 1996).

The fore- and hindlimb are connected to the body trunk by the shoulder and the pelvic girdles. Scapula and ilium are two dorsal components of the shoulder and pelvic girdles, respectively. Scapula is a triangular, large, thin bone that

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lies on the dorsolateral surface of the back. Scapular spine projects from the lateral surface of the body of the scapula, or scapular blade, and terminates with the acromion. This represents the more lateral part of the spine and articulates with the lateral part of the clavicle. The glenoid cavity or fossa of the scapula is located on the lateral extremity of the scapula and articulates with the head of the humerus. The coracoid process projects above the rim of the glenoid fossa.

Ilium consists essentially of a cranial ala or wing, of a narrow ramus, and of a caudal body that takes part in the formation of the acetabular cup. The latter articulates with the head of femur.

Traditionally, girdles belong to the limb field (Harrison, 1918), and local applications of fibroblast growth factors (FGFs) in the flank mesoderm of chick embryos (Cohn *et al.*, 1995; Crossley *et al.*, 1996), local treatment of regenerating limbs of amphibians with Retinoic acid (Maden, 1983), or systemic administration of Retinoic acid to early rodent embryos (Rutledge *et al.*, 1994) exert their influence on the proximal and distal elements of the appendicular skeleton, inducing the formation of complete supernumerary limbs accompanied with a duplication of the girdles. Nevertheless, the development of girdles is not influenced by any known signaling systems that pattern the rest of the limb along the three axes. The apical ectodermal ridge (AER) is a thickened epithelium at the tip of the limb bud that patterns the limb along its proximodistal axis through the production of members of the FGFs family (Niswander *et al.*, 1993). These growth factors exert their influence on the actively proliferating mesenchyme directly underneath, or Progress Zone. *Wnt7a* is expressed in dorsal ectoderm of the limb bud and is involved in the regulation of dorsoventral limb pattern (Riddle *et al.*, 1995). The Zone of Polarizing Activity (ZPA) is another signaling center along the posterior border of the bud that controls the development of the limb along the anteroposterior axis, and the product of *Sonic hedgehog* is the likely mediator of this activity (Riddle *et al.*, 1993). Removal of the AER causes proximal truncations in limb development that are more extensive at earlier stages of development but that never affect the development of the girdles (Saunders, 1948). The girdles develop normally also in the chick mutant *limbness* (Prahlad *et al.*, 1979). These mutants lack the rest of the wings and legs in association with abnormal development of the AER and absence of *Sonic hedgehog* activity (Ros *et al.*, 1996).

Gene knock-out experiments have shown that mutations in the *HoxA* and/or *HoxD* complexes cause defects in the limbs, while the more proximal regions of the appendicular skeleton are not affected. From the expression pattern of *Pax1* (Timmons *et al.*, 1994) and *Hoxc6* (Sharpe *et al.*, 1988) in the anterior proximal region of the forelimb bud, it has been suggested that these genes might be involved in the formation of the proximal elements of the limb. Similarly, *Hoxc10* and *Hoxc11* (Nelson *et al.*, 1996; Peterson *et al.*, 1992; Peterson *et al.*, 1994), that have a comparable expression in the proximal hindlimb bud, might be involved in patterning the pelvic girdle. Nevertheless, only for *Pax1* has a role in patterning the scapula been demonstrated. *Pax1* homozygous mutant mice lack the acromion and part of the scapular spina (Wilm *et al.*, 1998). The knockout of *Ptx1*, a bicoid-related gene, has shown its involvement in patterning the hindlimb. The homozygous mutants lack ilium and knee cartilage and show underdevelopment of the long bones (Lanctot *et al.*, 1999).

Emx1 and *Emx2* are two homeobox-containing genes (Simeone *et al.*, 1992) that represent the mouse homologous of the *Drosophila empty spiracle* (*ems*) gene (Dalton *et al.*, 1989). *ems* directs the formation of the cephalic segments intercalary, antennal, and of the preantennal region in *Drosophila* embryos. These segments are missing in *Drosophila* mutants for *ems*; in particular, these mutants lack the antennal appendages (Cohen and Jurgens, 1990; Dalton *et al.*, 1989), a sense organ that can be considered an outgrowth of the body wall representing a diversification of insects limb pattern (Panganiban *et al.*, 1997). *Emx1* and *Emx2* are expressed in the cephalic region of mouse embryos with nested expression domains in the developing cerebral cortex (Simeone *et al.*, 1992). *Emx2* is expressed also in the primordia of the urogenital system and in the limb buds (Pellegrini *et al.*, 1997; Simeone *et al.*, 1992). *Emx2* homozygous mutants (*Emx2*$-/-$) die soon after birth for the absence of kidneys (Miyamoto *et al.*, 1997; Pellegrini *et al.*, 1996; Yoshida *et al.*, 1997). These mutants lack other components of the urogenital systems, like gonads and genital tracts, and show important alterations in the development of the brain cortex.

Since the expression pattern in the developing limb, we have analyzed the homozygous mutants looking for limb defects. Surprisingly, we have found that scapula and ilium are missing in *Emx2* homozygous mutant mice. We describe the analysis of the limb phenotype in *Emx2*$-/-$ mutants and correlate the contribution of *Emx2* with that of *Pax1* and *Hoxc6* in the development of the shoulder girdle.

MATERIALS AND METHODS

Generation of Emx2−/− Mutants

Emx2 gene knockout was obtained as described (Pellegrini *et al.*, 1996). Essentially, we have replaced the second and part of the third helix of the homeobox-containing gene *Emx2* with the neomycin resistance gene by homologous recombination in Embryonic Stem cells. The recombinant clones were used to generate chimeras by morulae aggregation and subsequent germ line transmission.

Genotype Analysis

The genotype determination was performed by standard genomic Southern blot using probe PR20 (Pellegrini *et al.*, 1996).

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In Situ Hybridization

Synthesis of RNA probes from *Emx2*, *Collagen II*, *Pax1*, and *Hoxc6* was performed using DIG RNA Labeling Kit (Roche Molecular Biochemicals) according to manufacturer's instructions. Embryos of 10.0, 10.5, 11.0, and 11.5 days post coitum (dpc) were recovered in PBS, genotyped, and fixed in a solution of 4% parformaldehyde in PBS overnight. Embryos for whole-mount *in situ* were dehydrated in a methanol series. Embryos used for *in situ* on sections were dehydrated in ethanol series, embedded in paraffin, and cut in 8-μm-thick sections on a rotary microtome. Whole-mount *in situ* hybridization and *in situ* hybridization to paraffin sections were performed as reported (Wilkinson and Nieto, 1993).

Whole-Mount Skeletal Preparation

Whole-mount skeletal preparations of 18.5 dpc fetuses were stained with Alcian blue and alizarin red in order to have the cartilage tissue in blue and the ossified tissue in red. The staining procedure was performed as reported (Kessel *et al.*, 1990).

Histological Analysis

Wild-type and homozygous mutant embryos at 13.5 dpc were genotyped, fixed in Bouin's, and embedded in paraffin. The embryos were cut on a rotary microtome, and the tissue slices were stained with hematoxylin and eosin.

RESULTS

Analysis of the Skeletal Defects in Emx2−/− Mutants

In order to study the defects of the appendicular skeleton, 18.5 dpc fetuses were collected and processed for further analysis. Skeletal preparations of wild-type and homozygous mutant mice were stained with alizarin red and Alcian blue to differentiate the bony and cartilage elements of the limb skeleton. The analysis of the skeletal preparations of *Emx2−/−* mice shows the complete absence of the scapula in the shoulder girdle (compare Fig. 1A with 1B). Nevertheless, the distal end of the acromion is present and articulates with the clavicula; the coracoid and the glenoid cavity develop normally, and the latter is normally articulating with the head of the humerus (Fig. 1B). Concerning the pelvic girdle, *Emx2−/−* mice lack the major part of the ilium. The small remnant is represented by the acetabular cup articulating with the head of the femur and by a short iliac crest with an abnormal bifid aspect (compare Fig. 1C with 1D). At this level, sacral vertebrae have a reduced or absent surface articulating with the remnants of the ilium since the transverse processes of sacral vertebrae do not form the sacro-iliac joint (Fig. 1D).

We have analyzed 15 homozygous mutants and all the animals showed the reported skeletal defects.

Comparative Expression Analysis between Emx2, Collagen II, Pax1, and Hoxc6 in Developing Limb

In order to investigate the cause of the agenesis of the scapula, we have correlated the expression pattern of *Emx2* with that of *Collagen II (Col2a1)*, *Pax1*, and *Hoxc6* in the limb buds. The mRNAs of these genes have been localized by *in situ* hybridization in whole embryos and in transverse tissue sections of mouse embryos between 10.0 and 11.5 dpc. *Col2a1* probe marks the mesenchymal condensations and the cartilage primordia of the developing scapula and ilium. *Pax1* and *Hoxc6* are transcribed in the scapular region and have been experimentally associated to shoulder girdle development.

Whole-mount *in situ* hybridization at 10.5–11.0 dpc shows that *Emx2* transcripts are localized in the anterior and dorsal proximal region of the forelimb bud and at the base of the hindlimb bud (Fig. 2A). These regions are fated to give rise to the scapula in the shoulder girdle and to the ilium in the pelvic girdle (Muneoka *et al.*, 1989; Prahlad *et al.*, 1979; Vargesson *et al.*, 1997). *Emx2* probe marks also a line in the flank mesenchyme adjoining the fore- and the hindlimb buds and corresponding to the Wolffian ridge, a band of tissue from which the limb buds grow out (Fig. 2A). At 11.5 dpc, *Pax1* expression domain is partially overlapping the expression area of *Emx2* in the proximal anterior region of the forelimb bud and along the Wolffian ridge (compare Fig. 2B with 2A). In *Emx2−/−* mutant embryos of 11.5 dpc, *Pax1* expression area is enlarged and extends dorsally in the direction of *Emx2* domain (Fig. 2C). We have localized *Hoxc6* mRNAs at the level of the scapulo-humeral region (Fig. 2D). This localization is possibly coincident with the glenoid cavity and scapulo-humeral joint. In wild-type embryos at 11.5 dpc, the precartilaginous condensed mesenchyme of the developing scapula is marked by *Col2a1* (Fig. 2E). The scapula is not developing in *Emx2−/−* mutants as shown in wild-type embryos of the same stage probed with *Col2a1* (Fig. 2F).

More detailed information has been obtained by *in situ* hybridization on transverse tissue sections of limb bud between 10.0 and 11.5 dpc. The expression patterns of *Emx2*, *Pax1*, and *Hoxc6* have been compared to that of *Col2a1* in order to localize the transcripts of these genes temporally and spatially in respect to the forming scapula and humerus.

At 10.0–10.5 dpc, *Col2a1* probe marks the precartilaginous condensed mesenchyme of limb skeleton (Fig. 3A). In an alternate serial section, *Hoxc6* signals are localized at the level of the scapulo-humeral region (Fig. 3B). *Emx2* expression is restricted to a more proximal area, at the base of the limb, identifying the developing scapula (Fig. 3C). At 10.5–11.0 dpc, *Pax1* (Fig. 3D) and *Emx2* (Fig. 3E) domains of expression are partially overlapping anteriorly in the direction of the developing acromion (compare Fig. 3D with 3E). In an embryo of the same stage, a transversal section through the four limb buds shows that *Emx2* mRNAs are localized in the proximal part of the fore- and hindlimb

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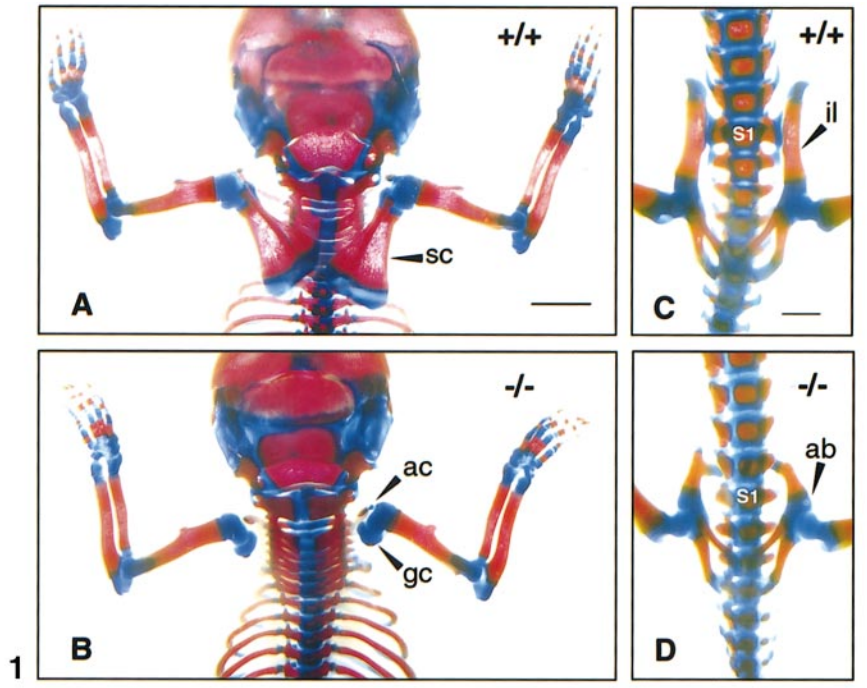

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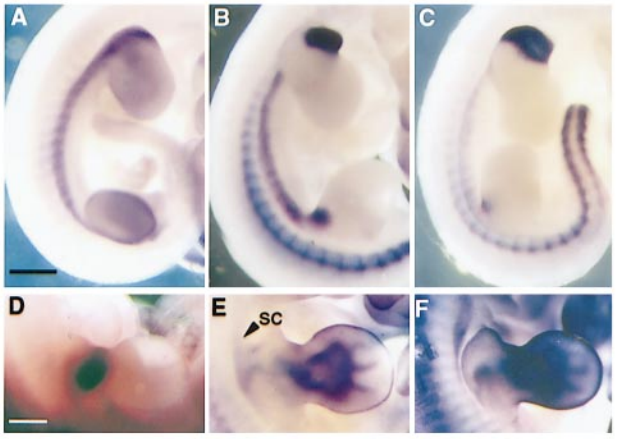

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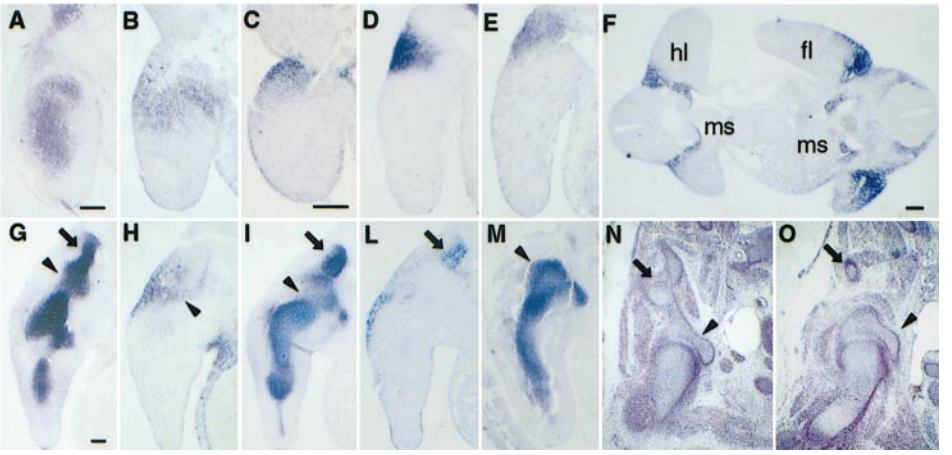

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buds (Fig. 3F). An intense expression is localized in the mesonephros underneath where urogenital system will form. At 11.5 dpc, the precartilaginous model of the scapula and of glenoid cavity/scapulo-humeral joint are well delineated by *Col2a1* probe (Fig. 3G). In an alternate serial section, *Hoxc6* probe demarcates only the area corresponding to the glenoid cavity/scapulo-humeral joint (Fig. 3H). Alternate serial sections probed with *Col2a1* (Fig. 3I) and *Emx2* (Fig. 3L), respectively, show that *Emx2* signals are restricted to the developing scapula. This description demonstrates that *Emx2* expression pattern is similar to that of *Col2a1* in the prechondrogenic condensed mesenchyme of the scapula (compare Fig. 3L with 3I) and that the anterior dorsal pattern of expression of *Pax1* and *Hoxc6* in the proximal limb bud is restricted to the acromio-clavear and scapulo-humeral/glenoid cavity regions, respectively. The precartilaginous mesenchyme of the scapula is absent in sections from *Emx2−/−* mutant embryos of 11.5 dpc where *Col2a1* probe marks only the condensed mesenchyme corresponding to the developing glenoid cavity (Fig. 3M). Tissue sections from wild-type and homozygous mutant embryos at 13.5 dpc have been stained with hematoxylin and eosin in order to study the morphology of the developing scapula. We have found that, in *Emx2−/−* mutants, the cartilage model of the scapula is not developing as compared to wild-type embryos (compare Fig. 3N with 3O). Furthermore, in the mutants, the glenoid cavity is normally developing, and a small remnant, corresponding to a piece of the medial vertebral border of the scapula, is barely visible.

DISCUSSION

The knowledge about girdle development is rather incomplete and only for *Pax1* has a specific role been pointed out in the formation of the acromion of the scapula. Scapula and ilium belong to the shoulder and pelvic girdles, they are both dorsally located, and are in connection with the stylopodium of the fore- and hindlimb, respectively. We have found that scapula and ilium are patterned by the same gene. These findings show for the first time that pelvic and scapular girdles are genetically linked likewise the fore- and the hindlimb in respect to *Hox* genes.

FIG. 1. Whole-mount skeletal preparations of wild-type and *Emx2−/−* mutant mice. The skeletons of fetuses at term of wild-type and *Emx2−/−* homozygous mutants have been stained with Alcian blue and alizarin red in order to differentiate the cartilage and the bony components of the girdles. (A) The scapula is present only in wild-type animals. (B) *Emx2−/−* fetuses lack the scapula. The glenoid cavity and the acromion are normally represented and articulate, respectively, with the head of humerus and the clavicle. (C) In wild-type animals, ilium is a component of the pelvic girdle. (D) In *Emx2−/−* skeletal preparations, ilium is almost totally absent. The only remnants are the acetabular cup that articulates with the head of femur and a reduced iliac crest with a bifid aspect. The transverse processes of sacral vertebrae do not fuse to form the articular surface in connection with the remnants of the ilium. Abbreviations: ab, acetabular cup; ac, acromion; gc, glenoid cavity; il, ilium; sc, scapula; S1, first sacral vertebra. Scale bar: 2 mm (A and B), 1 mm (C and D).

FIG. 2. Whole-mount *in situ* hybridization with *Emx2*, *Pax1*, *Hoxc6*, and *Col2a1* probes. (A) At 10.5–11.0 dpc, *Emx2* mRNAs are detected at the base of the buds and along the flank mesenchyme adjoining the two buds or Wolffian ridge. Signals in the proximal anterior and dorsal region of the forelimb bud demarcate the developing scapula. (B) At 11.5 dpc, *Pax1* expression overlaps partially *Emx2* expression in the proximal anterior region of the forelimb bud and along the Wolffian ridge. (C) *Pax1* expression in *Emx2−/−* mutants of the same age extends more dorsally in the direction of *Emx2* domain. (D) At 11.0 dpc, *Hoxc6* expression is localized at the level of the scapulo-humeral region. (E) In wild-type embryos at 11.5 dpc, the developing scapula and limb skeleton is marked by *Col2a1* probe that evidences the precartilaginous condensed mesenchyme. (F) The mesenchymal condensation corresponding to the scapula is not developing in *Emx2−/−* embryos of the same stage probed with *Col2a1*. Abbreviation: sc, scapula. Scale bar: 500 μm (A, B, C, E, and F), 500 μm (D).

FIG. 3. Comparative expression analysis of *Emx2*, *Pax1*, *Hoxc6*, and *Col2a1* in transverse sections of the developing limb and shoulder girdle. The mRNAs are localized by *in situ* hybridization in limb transverse tissue sections of embryos between 10.0 and 11.5 dpc. (A–C) At 10.0–10.5 dpc, *Col2a1* probe identifies the mesenchymal condensations of the appendicular skeleton (A). In an alternate serial section, *Hoxc6* trancripts are localized in a region most probably corresponding to the scapulo-humeral joint (B), while *Emx2* expression (C) is restricted to a more proximal area, representing the mesenchymal condensation of the developing scapula. (D and E) At 10.5–11.0 dpc, the expression domains of *Pax1* (D) and *Emx2* (E) are overlapping partially anteriorly in the direction of the developing acromion. (F) At 10.5–11.0 dpc, *Emx2* mRNAs are shown in the proximal part of the forelimb and hindlimb buds and in the mesonephros underneath. (G–I, L, and M) At 11.5 dpc, *Col2a1* probe defines the precartilaginous model of the scapula (arrow) and of the glenoid cavity/scapulo-humeral joint (arrowhead) (G). In a parallel section, *Hoxc6* transcripts are present only the area that will give rise to the glenoid cavity/scapulo-humeral joint (arrowhead) (H). From the comparison of alternate serial sections probed with *Col2a1* (I) and *Emx2*, it is evident that *Emx2* signals (L) are restricted to the developing scapula (arrow). The signals corresponding to the scapula are absent in sections from *Emx2−/−* mutant embryos where *Col2a1* probe (M) shows only the condensed mesenchyme corresponding to the future glenoid cavity (arrowhead) and to the distal part of the limb. (N and O) Hematoxylin and eosin-stained tissue sections (cut at the level of the glenoid fossa) from wild-type and homozygous mutant embryos at 13.5 dpc. The cartilage model of the scapula with the glenoid cavity articulating with the head of the humerus is evident only in wild-type embryos (N). In *Emx2−/−* (O), the major part of the scapula is absent, while the glenoid cavity is normally developing (arrowhead). Note that, in the mutant, a small part of the scapula is visible, corresponding to a piece of its medial vertebral border (arrow). Abbreviations: fl, forelimb buds; hl, hindlimb buds; ms, mesonephros. Scale bar: 150 μm (A, B, D, E), 150 μm (C), 150 μm (F), 150 μm (G–I, L–O).

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At 10.5 dpc, *Emx2* transcripts are located at the base of the forelimb bud in a dorsal and anterior localization. Later, at 11.5 dpc, the expression pattern identifies precisely the precartilaginous condensed mesenchyme of the scapula. This is more evident when the reciprocal relationships between scapula, humerus, and scapulo-humeral joint are well delineated by the *Col2a1* probe. In *Emx2*−/− mutants, the mesenchymal condensation corresponding to the scapula is not forming at 11.5 dpc, and, consequently, the cartilage model will not develop. At birth in these mutants, the scapula is absent, while, in wild-type animals, the bone is already partially ossified. The expression pattern and the effects of the knockout favor the hypothesis that *Emx2* is directly involved in patterning the scapula. The comparative expression analysis of *Emx2* and *Collagen II* genes with that of *Pax1* and *Hoxc6* contributes to further elucidate their role in the development of the scapula. The anterior dorsal pattern of expression of *Pax1* and *Hoxc6* in the proximal limb bud at 10.0–11.0 dpc is later restricted to the acromio-clavear and scapulo-humeral/glenoid cavity regions, respectively. At 11.0 dpc, *Pax1* expression pattern is overlapping partially the expression domain of *Emx2* in the anterior region of the limb bud fated to give rise to the scapular spine and to the acromion. In *Emx2* homozygous mutants, *Pax1* expression is enlarged and extends anterodorsally toward the expression domain of *Emx2*. The described pattern and its modifications in the mutants suggest an interaction between *Pax1* and *Emx2* in the formation of the spine of the scapula. *Pax1* might intervene in a first phase controlling mesenchymal cells proliferation (Ebensperger *et al.*, 1995), while, in a successive phase of cartilage differentiation, it may be necessary that *Emx2* control negatively *Pax1* expression. According to these data, *Emx2* patterns the scapula in association with *Pax1* and possibly *Hoxc6*. In particular, *Emx2* controls the formation of the scapular blade that constitutes the major part of the bone, *Emx2* and *Pax1* together might participate in the formation of the scapular spina, *Pax1* patterns the end of acromion (Wilm *et al.*, 1998), while *Hoxc6* is probably involved in the formation of the coracoid and of the glenoid cavity. Indirect evidence of *Hoxc6* participation in shoulder formation comes from experiments in chick embryos in which local application of Retinoic acid in the anterior and proximal mesoderm of the forelimb bud can expand *Hoxc6* gradient of expression and is accompanied with malformations and duplications of the shoulder girdle mainly affecting the coracoid and the head of the scapula (Oliver *et al.*, 1990). The normal development of the end of the acromion, of the coracoid process, and of the glenoid cavity in the mutants represents a further support of this assumption.

In the hindlimb, *Emx2* expression is localized at the base of the bud and later in the ilium condensed mesenchyme, thus patterning this part of the pelvic girdle. As shown by the skeletal preparations at birth, *Emx2* homozygous mutants lack most of the ilium. The only remnants are represented by the part involved in the formation of the acetabular cup and by a very small iliac crest with an abnormal bifid aspect. At this level, sacral vertebrae have a reduced or absent surface articulating with the ilium. Since *Emx2* is not transcribed in sacral vertebrae, the reduction of the articular surface, formed by the fusion of their transverse processes, must be secondary or related to altered signals from the reduced ilium. Experiments carried out in amphibians have shown that the lateral process that connects the sacral vertebrae to the ilium is induced by the presence of the ilium (Perri, 1953). In the absence or reduction of the iliac bone and posterior limb, there is a reduction or absence of these lateral processes and the sacral vertebrae adopt a presacral phenotype (Perri, 1953). In *Emx2* homozygous mutants, the reduced ilium may exert a reduced inductive influence on the sacral vertebrae that consequently do not present the fused lateral processes forming the articular surface for ilium.

The initial development of the scapula is not influenced by mediators of the signaling centers that pattern the rest of the limb. Surgical removal of the Apical Ectodermal Ridge (AER), or its abnormal development in the chick mutant *limbness*, causes truncations or absence of limb development that do not alter the development of the girdles (Prahlad *et al.*, 1979). There are evidences that initial limb bud outgrowth is induced by axial structures, like the intermediate mesoderm or the derived mesonephros (Crossley *et al.*, 1996). *Emx2* is expressed in mesonephric tubules, but the agenesis of the scapula cannot be secondary to the disturbed mesonephric development of the mutants for different reasons. In *Emx2*−/− mice, the mesonephros and Wolffian ducts develop normally initially and only successively degenerate (Miyamoto *et al.*, 1997). Then, the localization of *Emx2* transcripts precisely in those structures fated to give rise to the scapula is in favor of a direct action of this gene in the appendicular skeleton. Retinoic acid induces *Hoxc6* expression in the mesenchyme of the limb bud, and, in a similar manner, it could be a mediator of a retinoid signaling center in the flank mesenchyme influencing *Emx2* transcription and scapula formation. An indication in this direction may be the agenesis of the scapula in RARα and RARγ double mutants (Lohnes *et al.*, 1994).

Hox genes confer a positional identity to different components along axial structures. Targeted inactivation of genes from *Hoxd* and *Hoxa* clusters produce restricted alterations of bony elements along the proximodistal axis of the limb and were expected to induce alteration in the more proximal components of the appendicular skeleton. Surprisingly, we have found that scapula and ilium are patterned by *Emx2*. The phenotype analysis of *Emx2* homozygous mutants suggests that this gene might be a component of a patterning system involved in organizing the more proximal regions of the appendicular skeleton. *Emx2* might be implicated in controlling the development of the most "cephalic" or "proximal" structures in the primary as well as in the secondary axes.

A final consideration concerns medical aspects. In the very rare familial pelviscapular dysplasia the radiological finding of bilateral agenesis of the wing of the scapula and

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bilateral hypoplasia of the ala of the ilium associated to other anomalies is described (Cousin *et al.*, 1982). Nevertheless, the complete agenesis of the scapula has never been reported in human syndromes, and this may be related to the fundamental role of *Emx2* in urogenital development and the consequential absence of kidneys and premature neonatal death in the homozygous condition.

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