Multiple roles of *Hoxa11* and *Hoxd11* in the formation of the mammalian forelimb zeugopod

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Summary

Mutations in the 5′ or posterior murine Hox genes (paralogous groups 9-13) markedly affect the formation of the stylopod, zeugopod and autopod of both forelimbs and hindlimbs. Targeted disruption of *Hoxa11* and *Hoxd11* or *Hoxa10*, *Hoxc10* and *Hoxd10* result in gross mispatterning of the radius and ulna or the femur, respectively. Similarly, in mice with disruptions of both *Hoxa13* and *Hoxd13*, development of the forelimb and hindlimb autopod is severely curtailed. Although these examples clearly illustrate the major roles played by the posterior Hox genes, little is known regarding the stage or stages at which Hox transcription factors intersect with the limb development program to ensure proper patterning of the principle elements of the limb. Moreover, the cellular and/or molecular bases for the developmental defects observed in these mutant mice have not been described. In this study, we show that malformation of the forelimb zeugopod in *Hoxa11/Hoxd11* double mutants is a consequence of interruption at multiple steps during the formation of the radius and ulna. In particular, reductions in the levels of *Fgf8* and *Fgf10* expression may be related to the observed delay in forelimb bud outgrowth that, in turn, leads to the formation of smaller mesenchymal condensations. However, the most significant defect appears to be the failure to form normal growth plates at the proximal and distal ends of the zeugopod bones. As a consequence, growth and maturation of these bones is highly disorganized, resulting in the creation of amorphous bony elements, rather than a normal radius and ulna.

Key words: Hox genes, Limb development, Limb defects, Chondrogenesis, Mouse

Introduction

In recent years, many of the genes that are involved in early patterning of the vertebrate limb in three dimensions have been defined (for reviews, see Mariani and Martin, 2003; Niswander, 2003; Tickle, 2003). The embryonic limb is first visible as a small bud that protrudes from the body and initially contains a presumed uniform mass of mesenchymal cells covered by a layer of ectoderm. The limb bud grows and lengthens along the proximodistal (PD) axis. Coincident with outgrowth, the molecular regulatory cascades that are responsible for patterning the limb elements along the PD, anteroposterior (AP) and dorsoventral (DV) axes are activated. The molecular processes that establish the three axes of the limb are beginning to be elucidated (for reviews, see Mariani and Martin, 2003; Niswander, 2003; Tickle, 2003). Factors required for outgrowth of the limb have also been identified (Lewandoski et al., 2000; Min et al., 1998; Moon and Capecchi, 2000; Sekine et al., 1999; Sun et al., 2002; Xu et al., 1998; Yamaguchi et al., 1999). In addition, molecules that control the growth of the cartilage elements of the limb and the differentiation of cartilage into bone have been characterized (de Crombrugghe et al., 2001; Kronenberg, 2003). The skeletal elements of the limb are formed through a process known as endochondral ossification in which condensations of mesenchymal cells differentiate into cartilage and with maturation are replaced by bone. The mesenchymal condensations that give rise to the skeletal elements are laid down progressively along the PD axis (Forsthoefel, 1963), with the first condensation giving rise to the most proximal element, the stylopod (i.e. humerus or femur) followed by two zeugopod condensations (i.e. radius and ulna in the forelimb, or tibia and fibula in the hindlimb). Finally, further condensation and segmentation yields the many skeletal elements of the autopod (i.e. the carpalts and tarsals, metacarpals and metatarsals, and digits). A conundrum in the field of limb development has been establishing where Hox genes fit into the molecular-genetic program guiding formation and patterning of the limb elements. Previous misexpression studies have shown that *Hoxc8*, *Hoxd11* and *Hoxd13* can affect chondrocyte proliferation or cartilage condensation size (Goff and Tabin, 1997; Yueh et al., 1998). Clearly Hox genes perform crucial roles in limb development as specific combinations of Hox gene mutations result in severely deformed principal elements. For example, in mice mutant for *Hoxa10*, *Hoxc10* and *Hoxd10*, or mutant for both *Hoxa11* and *Hoxd11*, formation of the femur or the radius and ulna are drastically affected, respectively (Fig. 1) (Davis et al., 1995; Wellik and Capecchi, 2003).

To gain insight into the roles of posterior Hox genes in the formation of the principal limb elements, we chose to concentrate our analysis on *Hoxa11/Hoxd11* double mutants as inactivation of only two Hox genes is required to disrupt the...
formation of the forelimb zeugopod. We show that these two Hox genes are not required to initiate the formation of the zeugopod condensations although even at early times these condensations are smaller in the double mutants than in controls. Furthermore, the differentiation program that initiates cartilage formation also occurs normally in these mutant embryos. However, maturation of the cartilage template is appreciably delayed. Most importantly, the two growth plates at the proximal and distal ends of the radius and ulna condensations are never properly established. As a consequence, although cells in the condensations proliferate, differentiate and even eventually mature, the growth and maturation of these elements is severely perturbed, resulting in the formation of grossly misshapen and vastly shortened bony elements.

Finally, an analysis of mice with one functional allele of either Hoxa11 or Hoxd11 (i.e. Hoxa11+/– Hoxd11+/– or Hoxa11+/– Hoxd11+/–; mice) reveals an additional role for Hoxa11 and Hoxd11 in postnatal growth of the forelimb zeugopod.

Materials and methods

Mice and histology

Male and female mice heterozygous for both the Hoxd11 mutation (Davis and Capecchi, 1994) and a Hoxa11-null allele (Boulet and Capecchi, 2002) were mated to generate double mutant embryos. The limb mutant phenotype of mice with three or four mutant Hoxa11 and Hoxd11 alleles is virtually identical to that reported for the Hoxa11 allele described previously (Davis et al., 1995). As both male and female mice with three or four mutant alleles are sterile, they could not be used to generate double mutant embryos, which are obtained at a frequency of one in 16 from the double heterozygote intercrosses. Limb staging was according to the system of Wanek et al. (Wanek et al., 1989), and double mutant embryos were compared with embryos of the same limb stage or the same crown rump length as noted in the description of each experiment.

Embryos were fixed in Bouins or 4% paraformaldehyde/PBS overnight, rinsed in 5% sucrose phosphate and embedded in paraffin wax according to standard procedures. Some embryos were processed in an MVP tissue processor (Ventana Medical Systems). Sections of 10 μm were mounted on slides for standard Hematoxylin and Eosin (H & E) staining.

Newborn skeletons were fixed in 95% ethanol, stained in Alcian Blue (in 76% ethanol/20% acetic acid) at 37°C for 2 days. After rinsing in 95% ethanol, skeletons were then treated with 1% KOH for 4 to 5 hours, stained with Alizarin Red in 2% KOH for 1 hour, and cleared successively in 20% glycerol/1% KOH, 50% glycerol/1% KOH and 95% glycerol.

Cell proliferation and apoptosis assays

For cell proliferation assays, pregnant females were injected with a 10 mM solution of BrdU in PBS (1 ml per 100 g weight), and embryos were collected 1 hour later. After fixation overnight in 4% paraformaldehyde in PBS at 4°C, embryos were embedded in paraffin wax. Sections (5 μm) were stained with an anti-BrdU antibody conjugated with alkaline phosphatase (Boehringer Mannheim). Enzyme activity was detected using Fast Red (Boehringer Mannheim) or NBT/BCIP as substrate. Sections developed with Fast Red were mounted in 90% glycerol and NBT/BCIP sections were counterstained with nuclear Fast Red or Eosin and mounted with Permount (Fisher Scientific).

For cell proliferation analysis of postnatal growth plates, 5 μm sections were incubated with the PCNA monoclonal antibody (Novocastra). Binding of the primary antibody was visualized using goat anti-mouse secondary and horse anti-goat tertiary antibodies, both conjugated with HRP. Enzyme reactions were carried out with DAB or Vector SG (Vector) as substrate, and sections were counterstained with nuclear Fast Red.

Apoptosis assays were performed on 10 μm paraffin wax sections of 4% paraformaldehyde fixed embryos using the in situ Cell Death Detection Kit, Fluorescein (Boehringer Mannheim) or the Apoptag kit (Onkor/Intergen).

Immunohistochemistry and in situ hybridization

An anti-type II collagen antibody (Developmental Studies Hybridoma Bank) was used to stain 3% acetic acid/ethanol-fixed (E12.5) or Bouins-fixed (newborn) paraffin wax-embedded sections. Goat anti-mouse secondary antibody (Jackson Immunoresearch) conjugated with HRP and Vector SG (Vector) were used to visualize primary antibody. The anti-type X collagen antibody was kindly provided by R. Haronen and B. Olsen. After incubation with biotinylated goat anti-rabbit secondary antibody and ABC reagent (Vector), enzyme reactions used Vector SG. Newborn sections stained with these antibodies were pretreated with hyaluronidase (1 mg/ml in PBS for 45 minutes at 37°C).

Embryos for in situ hybridization were fixed in 4% paraformaldehyde in PBS/0.1% Tween 20 at 4°C for 1-3 days. Whole-mount in situ hybridization was carried out as described previously (Boulet and Capecchi, 1996). Radioactive in situ hybridizations were performed on 6 μm paraffin wax sections using 33P-labeled RNA probes. Sections were pretreated with proteinase K, re-fixed, dehydrated and hybridized overnight at 55-56°C. Sections were washed, RNase-treated and dipped in emulsion according to standard protocols.

The Sox9 and Ihh probes were generated by PCR amplification of genomic mouse DNA. The Sox9 probe contains 388 nucleotides from exon 3 (Wright et al., 1995) and the Ihh probe contains 631 nucleotides from exon 3 (GenBank X76291). The Pthlh probe was

Fig. 1. Mutations in paralogous Hox genes cause dramatic disruptions of limb skeletal morphology. (A-D) Alcian Blue/Alizarin Red-stained limbs. Forelimbs of (A) control newborn and (B) Hoxa11/Hoxd11 double mutants; hindlimbs of (C) control and (D) Hoxa10/Hoxc10/Hoxd10 triple mutant newborn.
Hoxa11/Hoxd11 mutant limbs obtained from K. Lee, the Pthr probe was a gift from B. St-Jacques and K. Lee, the Bmp2 probe was obtained from B. Hogan, the Ptch probe was a gift from M. Scott, and Se-Jin Lee kindly provided the Gdf5 probe. The Fgf10 probe was obtained from D. Ornitz and C. Deng. The Fgf8 probe was transcribed from a 280 bp Pst to Sac fragment from exon 5 and the 3’ UTR.

Results

Mesenchymal condensations that give rise to the radius and ulna are initiated normally in Hoxa11/Hoxd11 double mutants

The mesenchymal condensations that give rise to the principal skeletal elements of the limb emerge in a PD order during limb bud outgrowth. The radius and ulna condensations form at the distal end of the humerus condensation at about E11.5 (limb stage 5-6) (Wanek et al., 1989). Initiation of these condensations occurs at the appropriate time and position in Hoxa11/Hoxd11 double mutants (Davis et al., 1995) (Fig. 2A,B). However, even at early times, the size and shape of these condensations are abnormal. This is particularly evident for the condensation that will give rise to the ulna, which is much shorter than in control littermates by E13.5 (Fig. 2C-F).

To determine whether reduced mitotic activity of cells within the condensations contribute to the smaller size of the double mutant radius and ulna condensations, cell proliferation was evaluated in Hoxa11/d11 double mutant embryos at E12.5 and E13.5. The percentage of cells showing BrdU incorporation over the entire radius condensation during these periods was comparable with that observed in controls. By contrast, double mutant ulna condensations at both stages showed lower percentages of BrdU incorporation relative to controls (11% versus 19% in the E12.5 specimen and 15% versus 22% in the E13.5 specimen). Additionally, fewer BrdU-positive cells were detected at the distal ends of both the radius and ulna condensations in the double mutants at E13.5 compared with control embryos (Fig. 2E-H, and data not shown).

The TUNEL assay was used to visualize apoptotic cells in forelimbs of E11.5, E12.5 and E13.5 embryos. The majority of apoptotic cells at E11.5 are observed in the ‘opaque patch’ located between the developing radius and ulna mesenchymal condensations. The number of apoptotic cells in this region was comparable in double mutant embryos and wild-type littermate controls (data not shown). By contrast, when embryos between E12.5 and E13.5 (stage 8-9 limbs) were examined, double mutant embryos displayed an increase in the total number of apoptotic cells relative to control littermates. Excessive...
TUNEL-positive cells were located in regions showing significant, but lower, levels of apoptosis in normal embryos (Fig. 2I-L).

Chondrogenesis appears to initiate normally in Hoxa11/d11 double mutants

The reduced zeugopod condensation size in double mutant embryos may also reflect a failure of discrete populations of mesenchymal cells to activate genes required to turn on the chondrogenic program in the absence of Hoxa11 and Hoxd11 function. The expression patterns of several genes involved in the establishment of prechondrogenic condensations were examined.

The Sox9 gene product is a transcription factor essential for chondrocyte differentiation, and is expressed in all chondroprogenitors (Ng et al., 1997; Zhao et al., 1997). Cells lacking Sox9 are unable to switch on expression of chondrocyte-specific genes or participate in the process of mesenchymal condensation (Bi et al., 1999). The Sox9 expression pattern in Hoxa11–/–Hoxd11–/– sections appears comparable with the controls, although the pattern reflects the reduced zeugopod condensation size characteristic of E12.5 mutant embryos (Fig. 3A-D and data not shown).

Subsequent to condensation, pre-chondrogenic cells initiate expression of type II collagen and become chondrogenic (Cheah et al., 1991). To determine whether the mesenchymal cells of the double mutant zeugopod make this transition, type II collagen expression was examined by immunohistochemistry. At 12.5 days of embryonic development, the pattern and level of type II collagen expression in double mutant radius and ulna condensations were similar to those seen in control condensations (Fig. 3E,F).

We also examined the expression patterns of Bmp2 and Gdf5, but were unable to detect any differences between control and double mutant embryos (data not shown).

Delayed forelimb bud outgrowth is associated with reduction of Fgfb and Fgfl expression

The forelimb buds of E9.5 and E10.5 double mutant embryos (limb stage 2-3) appear identical to those of control littermates (data not shown). At E11.5 (limb stage 5-6), forelimb bud appearance of some double mutants was distinguishable from control littermates of the same crown-rump length (Fig. 4A,B). By 12.5 days of gestation, there was an obvious delay in forelimb bud growth relative to littermate control embryos, particularly with respect to development of the handplate (Fig. 4C,D). By E13.5, double mutant forelimbs were readily distinguishable from normal littermates (Fig. 4E,F). The forearms were noticeably shorter and indentations between digits are slightly deeper in control embryo (arrows in E,F).
bud growth observed in Hoxa11/d11 mutants is the consequence of a change in Fgf8 or Fgf10 expression. The pattern and level of Fgf8 expression in double mutant embryos at E9.5 was very similar to that of control littermates (data not shown). In 10.5-day-old embryos (limb bud stage 3), the region of Fgf8 expression appeared narrower in the double mutants than in controls (Fig. 5A,B). At E11.5 (stage 4-5 limb buds) Fgf8 mRNA in the anterior AER of double mutant embryos was noticeably less abundant (Fig. 5C,D). The expression pattern of Fgf10 at 11.5 was also altered in the double mutant embryos, with a considerable reduction in the levels of transcript in the anterior progress zone mesenchyme (Fig. 5E,F).

To determine if the delay in expansion of the double mutant distal forelimb bud to form a handplate is due to decreased cell proliferation, we compared BrdU incorporation into cells of the progress zone of double mutant and control E11.5 embryos. No reproducible differences could be detected between double mutants and controls (data not shown).

Chondrocyte maturation is severely delayed in Hoxa11/d11 double mutant radius and ulna

After perichondrium formation, chondrocytes in the center of each cartilage anlage begin to undergo the process of maturation. Prehypertrophic cells differentiate at the center of the condensation and subsequently mature to form hypertrophic cells. Growth plates that form at the ends of long bone cartilage precursors consist of ordered arrays of cells (i.e. in reserve, proliferating, prehypertrophic and hypertrophic zones) (Poole, 1991). As shown previously, condensed mesenchyme that gives rise to the radius and ulna of Hoxa11/d11 double mutant embryos makes the initial transition to a type II collagen-expressing chondrocyte fate. However, histological examination of the double mutants demonstrated that chondrocytes in the radius and ulna fail to mature in a normal manner. In the double mutant at E16.5, chondrocytes in radius and ulna condensations showed a uniform appearance with no evidence of hypertrophic cells, whereas chondrocytes in the humerus and digits progress normally through their maturation pathway (Fig. 6A,B and data not shown).

Even in the absence of overt differentiation at this time, the radius and ulna cartilage elements of Hoxa11/d11 mutant embryos continue to increase in size. At E16.5, dividing cells in wild-type embryos are confined to proliferative zones of the growth plates at the proximal and distal ends of the radius and ulna. In double mutant embryos, cells that incorporate BrdU were found throughout the radius and ulna (data not shown). At this stage, there were no significant differences in the percentage of proliferative cells in the entire double mutant radius or ulna compared with the control. In spite of continued proliferation, the tremendous extension in length accompanying chondrogenic hypertrophy in wild-type embryos does not occur in the double mutants. Instead, what is observed is disorganized cell growth, delayed chondrocyte differentiation and maturation with limited bone formation in the center of these normally long bones.

Expression of Ihh is perturbed in Hoxa11/d11 double mutant embryos

To determine the nature of the block in chondrocyte development, we assayed the expression of a number of genes that are transcribed during the course of chondrocyte maturation. Type X collagen is specifically expressed in chondrocytes that
have undergone hypertrophy (Schmid and Linsenmayer, 1985). At E16.5, no type X collagen could be detected in the radius and ulna of the double mutants (Fig. 6D). Strong staining for type X collagen was visible in the zeugopod of control embryos at this stage and in the humerus and digits of both controls and double mutants (Fig. 6C,D and data not shown).

Indian hedgehog plays crucial roles in the regulation of chondrocyte proliferation and maturation (Karp et al., 2000; St-Jacques et al., 1999). Initially, Ihh is expressed in the central region of each long bone cartilage element. At E12.5, transcripts of both Ihh and patched (Ptch), the Ihh receptor, were detected in the radius and ulna condensations of Hoxa11/Hoxd11 double mutant embryos, but the levels of expression of Ihh and Ptch were significantly reduced relative to littermate controls (Fig. 7A,B and data not shown). Subsequently, Ihh expression is progressively restricted to postmitotic prehypertrophic chondrocytes in growth plates at the proximal and distal ends of long bones (Bitgood and McMahon, 1995; Iwasaki et al., 1997; Vortkamp et al., 1996). At E15.5 (stage 12 of forelimb development) and E16.5, Ihh transcripts could not be detected in double mutant radius and ulna (Fig. 7C,D and data not shown).

Pthlh (parathyroid hormone-like peptide) is a paracrine factor that is required for the proper regulation of chondrocyte maturation (Wallis, 1996), and the Pthlh gene is transcribed in cells of the perichondrium with higher levels in the periarticular regions (Lee et al., 1996). In the absence of Pthlh or its receptor, chondrocytes closer to the articular ends of each element differentiate precociously, resulting in the production of shorter and wider bones (Karaplis et al., 1994; Lanske et al., 1996). Double mutant embryos at 15.5 days of gestation showed greatly reduced levels of Pthlh mRNA in the perichondrium of the distal radius and ulna, while the hybridization signal surrounding the carpal elements appeared similar to the control (Fig. 7E,F).

Finally, Pthr, the receptor for Pthlh, is normally expressed in the prehypertrophic cells of the growth plate (Lee et al., 1996). Transcripts for Pthr can be detected in the radius and ulna of wild-type embryos as early as E13.5 (stage 10, Fig. 7G). However, in Hoxa11/d11 double mutants at the same limb stage, no Pthr signal could be seen in the zeugopod (data not shown). Pthr mRNA was still undetectable in the double mutant radius and ulna at E16.5, although Pthr expression was readily detectable in the digits of double mutants at this stage.
of chondrocyte maturation, we examined the expression patterns or molecular signs of chondrocyte maturation.

Double mutant radius and ulna show no detectable histological (Fig. 7H). Therefore as late as 16.5 days of gestation, the double mutant radius and ulna show no detectable histological or molecular signs of chondrocyte maturation.

To facilitate interpretation of the observed defects in chondrocyte maturation, we examined the expression patterns of Hoxa11 and Hoxd11 in mid-gestation mouse embryos. During the early stages of chondrocyte maturation in the radius and ulna, Hoxa11 and Hoxd11 transcripts are detected in cells surrounding the radius and ulna condensations (Fig. 7J). Expression in chondrocytes was not detectable above background.

Although no molecular evidence of chondrocyte maturation was evident at E16.5, a few cells that resemble hypertrophic chondrocytes were discernible in the center of the radius and ulna of newborn Hoxa11/d11 double mutant specimens (Fig. 8A,B,E,F). Small areas of mineralization in the centers of both skeletal elements were revealed by Alizarin Red staining (Fig. 1). Furthermore, Ihh and Pthr transcripts, and type X collagen, were also readily detected in these aberrant maturation centers (Fig. 8C,D,G,H and data not shown).

Reduced levels of Hoxa11 and Hoxd11 affect postnatal bone growth

The radius and ulna of adult mice homozygous for either Hoxa11 or Hoxd11 and heterozygous for a mutant allele of the other paralogue (Hoxa11+/–/Hoxd11+/– or Hoxa11+/–/Hoxd11–/–) are ~50% shorter than those of wild-type animals of the same age (Davis et al., 1995). Surprisingly, no detectible abnormalities in embryonic limb growth and differentiation were detected in mice with three mutant alleles during early to mid-gestation, up to at least E14.5. In addition, the zeugopod bones in newborn mice were only slightly shorter than controls (Fig. 9A,B). In the forelimb of a 2-week-old (P14) Hoxa11+/–/Hoxd11+/– animal, the distal growth plates of both radius and ulna were significantly shorter than those of a control littermate (Fig. 9C,D and data not shown). In addition, the secondary ossification centers of the distal ends of each element were greatly reduced in size and showed delayed ossification relative to the control, which showed complete mineralization by this time. A reduction in growth plate length was also readily apparent in newborn specimens (PO) (Fig. 9E,F). Although the zeugopod bones of adult Hoxa11 homozygous mice are somewhat shorter and thicker than wild-type bones (Boulet and Capecchi, 2002; Small and Potter, 1993), newborn Hoxa11–/– specimens did not have shortened growth plates analogous to those seen with three mutant alleles (data not shown).

Cell proliferation was examined in newborn and one-day postnatal (P1) growth plates using an antibody directed against PCNA (proliferating cell nuclear antigen). No consistent alteration in the percentage of cells labeled with PCNA antibody was detected. However, the density of cells in the proliferation zone of the growth plate was reduced relative to controls (averages of 12% and 25% reductions for newborn radius and ulna and 16% and 26% for P1 radius and ulna, respectively). No increase in the number of cells undergoing programmed cell death was detected (data not shown).

Expression of Ihh (Fig. 9G,I) and Pthr (Fig. 9H,J) in the Hoxa11+/+Hoxd11+/+ newborn radius and ulna were similar to that observed in controls.

The expression patterns of Hoxa11 and Hoxd11 at late embryonic stages and in newborn forelimb have not previously been reported. In situ hybridization detected Hoxa11-expressing cells surrounding the distal ends of the zeugopod cartilages up to E16.5, while Hoxd11 transcripts could still be distinguished in a similar pattern at E16.5 (Fig. 9K) and E17.5. However, expression of neither Hoxa11 nor Hoxd11 could be detected above background in newborn forelimbs (data not shown).

Discussion

In this study, we show that multiple aspects of forelimb development are affected when the products of Hoxa11 and Hoxd11 are removed from the mouse embryo by targeted mutagenesis. The observed delay in limb bud growth correlates with decreased expression of Fgf8 and Fgf10, factors known to be required for the outgrowth of the limb bud (Lewandoski et al., 2000; Moon et al., 2000; Moon and Capecchi, 2000;
Sekine et al., 1999). In addition at early times during limb skeletal development, reduced cell proliferation in the ulna condensation and at the distal ends of both the ulna and radius condensations, as well as an increase in the number of apoptotic cells were detected in the forelimb bud. All of these factors may have contributed to the formation of extremely small ulna condensations and undersized radius condensations in Hoxa11/Hoxd11 double mutants. Subsequently, chondrocytes in the mutant condensations fail to undergo maturation at the appropriate developmental stage. No signs of chondrocyte hypertrophy were detectable until 5-6 days after wild-type chondrocytes had begun to mature.

However, the most informative finding is that formation of growth plates at each end of the radius and ulna condensations is extremely delayed. The enormous growth extension of these long bones along the proximodistal axis is crucially dependent on the presence of growth plates at both ends of the condensation. Normally, chondrocyte proliferation and maturation is restricted to the growth plates and longitudinal growth relies on the appropriate balance of several signaling molecules and their receptors within the growth plate. Ihh expression is initially weak in the double mutants and disappears completely before normal localization to separate domains at each end of the condensations occurs. Although the percentage of cells incorporating BrdU in Hoxa11/Hoxd11 double mutants at E16.5 was similar to that observed in control embryos, the spatial distribution of dividing cells was very different. BrdU-positive nuclei were randomly distributed within the radius and ulna condensations, whereas with control embryos, dividing cells were largely restricted to the ends (i.e. within the growth plates). In the absence of growth plates resulting from the enormous delay in chondrocyte maturation, chondrocytes continue to proliferate throughout the condensation in a disorganized manner, and grossly misshapen and shortened bony elements are produced instead of the normal radius and ulna. Extremely delayed formation of abnormal growth plates at the ends of the zeugopod elements in the double mutants suggests that an important function of these Hox genes is to refine the pattern of the zeugopod cartilage elements to ensure appropriate growth of the bones along the proximodistal axis. Further experiments would be required to determine unequivocally whether defective growth plate formation is directly due to the absence of Hoxa11 and Hoxd11 or a secondary consequence of reduced condensation size.

Finally, abnormal growth plate architecture is evident in mice containing only a single functional allele of Hoxa11 and Hoxd11 (i.e. Hoxa11+/–Hoxd11–/– or Hoxa11–/–Hoxd11+/– mice). The radius and ulna of adult mice of these genotypes are approximately half as long as in wild-type littermates.
(Davis et al., 1995). However, the discrepancy in the length of these long bones relative to controls becomes evident primarily post-birth, suggesting the importance of establishing the correct growth plate cellular architecture in allowing normal postnatal growth of these bones. The observed reduction in cell density is in contrast to the effect of mutations in the pRB related genes, p130 and p107 (Cobrinik et al., 1996), which are required for the cell cycle withdrawal that accompanies chondrogenic differentiation, and could indicate that cells are exiting the cell cycle or differentiating prematurely.

Previous studies on the functions of Hox genes in limb development fall into two categories: loss-of-function and gain-of-function. By combining loss-of-function alleles for two or three Hox genes, we now know that the most 3’ members of the posterior Hox gene (Abd-B) group (e.g. Hox9 and Hox10 paralogs) are crucial for proper development of the most proximal limb skeletal element, the stylopod, while Hox11 paralogous genes function mainly in patterning the zeugopod, and the 5’ genes, the Hox13 paralogs, function predominantly in specification of the most distal structures, the elements of the autopod (Davis et al., 1995; Fromental-Ramain et al., 1996a; Fromental-Ramain et al., 1996b; Wellik and Capecci, 2003). However, little could be learned regarding mechanisms of Hox gene function by simple examination of the mutant phenotypes. More thorough studies have hinted at effects on multiple processes. For example, while heterochrony or a change in the rate and timing of cartilage formation was seen in Hoxd13 mutant limbs (Dollé et al., 1993), a loss of cell adhesion and chondrogenic capacity was discovered in Hoxa13 mutant limb bud cells (Stadler et al., 2001). Gain-of-function studies point to potential roles for Hox genes in control of proliferation of undifferentiated limb bud mesenchymal cells, as well as cells in the proliferative zone of developing cartilage (Goff and Tabin, 1997; Morgan and Tabin, 1994). The result of controlled overexpression of Hoxc8 suggests that Hox genes have the capacity to regulate chondrocyte differentiation (Yueh et al., 1998). Our analysis of Hoxa11/Hoxd11 double mutant limbs confirms multiple roles for Hox genes in patterning of the limb skeleton. Not only did we find that Hox genes are required for the proper level of cell proliferation, as evidenced by delayed limb bud outgrowth and reduced BrdU incorporation, but, in addition, we have obtained further evidence supporting a role for Hox genes in the control of chondrocyte differentiation, in particular in the formation of normal growth plates at the ends of the radius and ulna cartilage elements.

Effects on the expression patterns of Fgf8 and Fgf10, molecules known to be crucial for limb outgrowth, were identified in double mutant forelimb buds. Fgf10 mutant mice completely lack limbs (Min et al., 1998; Sekine et al., 1999). In the absence of Fgf8 expression, limb bud outgrowth is delayed and some skeletal elements are reduced in size or completely absent (Dollé et al., 1989; Lewandoski et al., 2000; Moon and Capecci, 2000). Prior to E10.75, the expression patterns of Hoxa11 and Hoxd11 overlap in the progress zone (Davis and Capecci, 1994; Dollé et al., 1989; Haack and Gruss, 1993; Small and Potter, 1993). Because Hoxa11 and Hoxd11 are expressed in the mesenchyme, but not in the ectoderm of the limb bud, we presume that decreased Fgf10 expression is more likely to be the primary effect. The reduced expression of Fgf8 in the anterior AER would then be a secondary consequence of reduced Fgf10 expression in the anterior distal mesenchyme (Min et al., 1998; Ohuchi et al., 1997; Sekine et al., 1999). The effects on the anterior regions of both expression patterns do not correlate obviously with any aspect of the skeletal phenotype of Hoxa11/Hoxd11 double mutants. However, reduced Fgf expression levels may translate into a more wide-ranging effect on limb outgrowth or may reflect a yet to be determined effect on the function of another crucial limb bud pathway. Previous results on the effects of mis-expression of Hoxd11 (Goff and Tabin, 1997; Morgan and Tabin, 1994) and Hoxd12 (Knezevic et al., 1997) might be partially explained in terms of an increase in Fgf10 expression in the anterior limb bud which could, in turn, lead to enhanced growth and additional condensations (as observed after elevated levels of bFgf are induced in the chick wing (Riley et al., 1993). However, no effect on Shh expression was detected in Hoxa11/Hoxd11 double mutants at E9.5 or E10.5 (data not shown).

Analysis of Ihh mutants demonstrated that, in addition to a Pthlh-dependent role in the control of chondrocyte maturation, Ihh is also essential for normal chondrocyte proliferation (Karp et al., 2000; St-Jacques et al., 1999). In Ihh mutants, a 50% reduction in cell division in the humerus was seen between E12.5 and E14.5 (St-Jacques et al., 1999). Therefore, it seemed reasonable to postulate that the failure to maintain Ihh expression might contribute to the shorter radius and ulna of double mutants. However, the percentage of cells incorporating BrdU in the 16.5-day Hoxa11+/–/Hoxd11+/– radius and ulna was not reduced relative to wild-type growth plates. Therefore, although Ihh transcripts had been below detectable levels for at least 1 day, the mitotic activity of chondrocytes was not significantly altered. As Pthlh transcripts are undetectable at the articular surfaces of skeletal elements in Ihh+/+ animals (St-Jacques et al., 1999), the reduction in Pthlh expression seen in double mutants could be a secondary effect of the absence of Ihh. However, Pthlh transcripts are present in Wnt5a mutants in the absence of detectable levels of Ihh (Yang et al., 2003).

Development of secondary ossification centers at the distal ends of the radius and ulna is severely affected in mice with three mutant alleles. Ossification of the epiphyses is thought to occur by a distinct Ihh-independent pathway (Vu et al., 1998). Hoxa11 and Hoxd11 may play a specific role in the allocation of the population of cells destined to contribute to the secondary ossification centers or in the maturation of these cells, again resulting in the refinement of bone pattern.

In summary, it appears that Hoxa11 and Hoxd11 intersect the developmental pathway leading to forelimb zeugopod formation at multiple steps, from affecting the size and shape of the early condensations, to controlling the rate of chondrocyte maturation, and to ensuring that growth plates are properly positioned at the ends of the condensations. Mice with three mutant alleles further revealed a role of these genes in formation of the secondary ossification centers at the ends of these bones. Future experiments will be directed at determining the precise targets for Hox gene regulation in these pathways.

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