Hox11 genes regulate postnatal longitudinal bone growth and growth plate proliferation

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ABSTRACT

Hox genes are critical regulators of skeletal development and Hox9-13 paralogs, specifically, are necessary for appendicular development along the proximal to distal axis. Loss of function of both Hox11 and Hoxd11 results in severe malformation of the forelimb zeugopod. In the radius and ulna of these mutants, chondrocyte development is perturbed, growth plates are not established, and skeletal growth and maturation fails. In compound mutants in which one of the four Hox11 alleles remains wild-type, establishment of a growth plate is preserved and embryos develop normally through newborn stages, however, skeletal phenotypes become evident postnatally. During postnatal development, the radial and ulnar growth rate slows compared to wild-type controls and terminal bone length is reduced. Growth plate height is decreased in mutants and premature growth plate senescence occurs along with abnormally high levels of chondrocyte proliferation in the reserve and proliferative zones. Compound mutants additionally develop an abnormal curvature of the radius, which causes significant distortion of the carpal elements. The progressive bowing of the radius appears to result from physical constraint caused by the disproportionately slower growth of the ulna than the radius. Collectively, these data are consistent with premature depletion of chondrocytes in the growth plate of Hox11 compound mutants, and demonstrate a continued function for Hox genes in postnatal bone growth and patterning.

KEY WORDS: Hox genes, Growth plate, Chondrocyte, Postnatal skeletal development

INTRODUCTION

The proximodistal elongation of long bones occurs through the process of endochondral ossification within the growth plate. The growth plate is organized into distinct cellular zones: the reserve or resting zone (RZ), the proliferative zone (PZ), and the prehypertrophic/hypertrophic zone (HZ). Chondrocytes at the epiphyseal end of the growth plate make up the reserve zone. RZ chondrocytes are slow dividing, stem/progenitor cells which give rise to flattened columns of PZ chondrocytes (Abad et al., 2002). Chondrocytes in the PZ proliferate rapidly and undergo morphological changes to become oriented into columns of cells arranged parallel to the long axis of the bone (Hunziker, 1994). As cells in the columns reach the metaphyseal side of the growth plate and enter the HZ, it has been reported that chondrocytes exit the cell cycle, undergo hypertrophy and initiate apoptosis (Beier, 2005). Concomittantly, hypertrophic chondrocytes secrete significant amounts of extracellular matrix which becomes mineralized and provides a template for new bone formation (Mackie et al., 2011). The cartilage matrix of the growth plate is subsequently remodeled and replaced by bone through the function of osteoclasts and osteoblasts.

Longitudinal bone growth is regulated both locally and systemically by a host of hormones and growth factors that function to coordinate proliferation, matrix synthesis and differentiation of chondrocytes (Kronenberg, 2003; Wit and Camacho-Hubner, 2011). While the differentiation program of growth plate chondrocytes is similar in all long bones, elongation occurs at different rates for each element to achieve correct proportionate lengths for the adult animal (Wilsman et al., 1996b). These rates of growth can vary dramatically. Indeed, growth plates at opposite ends of the same long bone can differ by a factor of two- to three-fold (Wilsman et al., 1996b). These differences in growth rate contribute to final bone lengths and morphology, however the processes that govern these differential rates of growth are poorly understood.

Hox genes are a family of highly conserved, homeodomain-containing transcription factors crucial for axial and appendicular patterning. In the limb skeleton, Hox9-13 genes function to pattern the proximodistal axis and loss-of-function mutations of paralogous genes result in dramatic, region-specific perturbations of skeletal development. Loss of Hox9 and/or Hox10 genes results in mispatterning primarily of the stylopod element (humerus or femur) (Fromental-Ramain et al., 1996b; Wellik and Capecchi, 2003). Loss of Hox11 function leads to severely truncated zeugopod skeletal elements (radius/ulna or tibia/fibula) (Davis et al., 1995; Wellik and Capecchi, 2003; Boulet and Capecchi, 2004). In Hoxa13/Hoxd13 mutants, the autopod skeletal elements (hand or foot bones) are affected (Dollé et al., 1993; Fromental-Ramain et al., 1996a; Knosp et al., 2004). The genetic function of Hox factors during development is well documented, but continuing roles for these genes postnatally is less explored.

A high degree of functional redundancy exists between members of a Hox paralogous group. For example, single allele mutants of Hoxa11 or Hoxd11 exhibit minor developmental defects, which include fusion of various carpal bones, minor malformations of the distal epiphysisal end and a slight thickening of the radius and ulna (Small and Potter, 1993; Davis and Capecchi, 1994; Favier et al.,
demonstrate a continuing role for the radius and ulna contribute to an anterior bowing of the mutant ulna is more severely affected than the radius resulting in a PZ. Additionally, the longitudinal growth of the compound depleted in the mutant growth plate during postnatal growth is shortened in compound mutants. Chondrocytes are prematurely lengths being comparable to controls at birth, growth plate lengthHowever, during postnatal growth, the compound mutant radius compound mutants is indistinguishable from controls at birth. However, by adult stages, Hox11 compound mutant animals exhibit a significant reduction in zeugopod skeletal length (Davis et al., 1995). The purpose of this study was to define the morphological and cellular processes that contribute to the postnatal growth defects in Hox11 compound mutants during postnatal development.

We show that Hoxa11 and Hoxd11 continue to be expressed in the forelimb zeugopod throughout postnatal stages. Consistent with previous reports, the skeletal morphology of Hox11 compound mutants is indistinguishable from controls at birth. However, during postnatal growth, the compound mutant radius and ulna grow at slower rates than controls at all time points examined and growth arrests at earlier stages. Despite total bone lengths being comparable to controls at birth, growth plate length is shortened in compound mutants. Chondrocytes are prematurely depleted in the mutant growth plate during postnatal growth correlated with increased levels of proliferation in the RZ and PZ. Additionally, the longitudinal growth of the compound mutant ulna is more severely affected than the radius resulting in a bone that is disproportionately shorter. The altered proportions of the radius and ulna contribute to an anterior bowing of the compound mutant radius in adult animals. Together, these results demonstrate a continuing role for Hox11 genes in postnatal bone growth.

RESULTS

Hox11 genes remain expressed through postnatal and adult stages

We previously published detailed developmental expression analysis of Hoxa11 in the embryo utilizing a Hoxa11eGFP knock-in allele (Nelson et al., 2008; Swinehart et al., 2013). Embryonically, Hoxa11eGFP is expressed in the connective tissue elements of the forelimb including the perichondrium/periosteum, tendon and muscle connective tissue with the strongest expression surrounding the distal end of the radius and ulna (Swinehart et al., 2013). Utilizing this Hoxa11eGFP allele, we show that Hoxa11eGFP continues to be expressed at birth and through postnatal stages. Hoxa11eGFP is expressed strongly in the perichondrium and along the trabecular bone surface, with lower expression observed within the most distal RZ chondrocytes (Fig. 1A-D). Expression of Hoxd11 is observed in the connective tissue surrounding the distal radius and ulna in a pattern similar to Hoxa11eGFP expression (Fig. 1E-F and data not shown). qPCR analysis for both Hoxa11 and Hoxd11 on whole bones (radius and ulna) at E12.5, E18.5 and 1, 2, and 4 weeks of age demonstrates continued expression of both genes through adult stages. Interestingly, Hoxd11 shows higher relative expression at embryonic stages, but both Hoxa11 and Hoxd11 remain expressed through adult stages.

Abnormal postnatal skeletal growth in Hox11 compound mutants

To assess the development of the compound mutant skeletal phenotype during postnatal stages, we performed micro-computed tomography (µCT) analysis at 0, 2, 4, 6 and 8 weeks of age on animals with the genotype: Hoxa11−/−;Hoxd11−/−. The humerus was measured as an internal control for animal size as the length, bone quality, and bone morphology of the humerus and was indistinguishable between Hox11 compound mutants and controls (Fig. 2). Skeletal preparations and isosurface renderings of µCT scans allow visualization of the morphology of the radius and ulna of control and Hox11 compound mutants throughout postnatal growth (Fig. 3A). While no statistical difference in mineralized bone length is measurable at birth, the length of the ulna is trending towards being shorter in mutants compared to controls and slight morphological differences in the radius and ulna can already be observed (Fig. 3A-C). Abnormal curvature of the radius in compound mutants is readily apparent at two weeks of age and becomes progressively more severe through postnatal development. In addition, the distal radioulnar joint is dysmorphic and the radius of compound mutants progressively extends ectopically into the carpal bones (Fig. 3A, arrows). The overgrowth of the radius results in a forward subluxation of the hand and outwards rotation of the autopod.

To begin to dissect the onset of the postnatal phenotypes, the mineralized lengths of the radius and ulna were measured along the central curvature of the bone at 0, 2, 4, 6, and 8 weeks of age (Fig. 3A, solid and dashed lines). No significant differences in mineralized length of the radius or ulna was observed at newborn stages between controls and compound mutants, consistent with previous reports (Fig. 3B,C) (Boulet and Capecchi, 2004). Differences in bone length are observed early in postnatal development and by 2 weeks of age, both the radius and ulna of the compound mutant are shorter compared to controls: the radius is 15% reduced and ulna 20% reduced. By 4 weeks of age, the differences in length between the compound mutant and controls are exacerbated further (radius 17%, ulna 27% reduced). Longitudinal bone growth has slowed dramatically or stopped in all bones in both controls and mutants by 8 weeks of age, and at this stage the Hox11 mutant radius is 22% shorter and the ulna is 30% shorter than their analogous control counterparts (Fig. 3B,C).

In both controls and mutants, the rate of bone growth decreases with age. Consistent with the reduction in total bone length, the growth rate of both the mutant radius and ulna is reduced compared to controls at all time points examined (Fig. 3D, solid bars versus open bars). Interestingly, the rate of growth of the mutant ulna slows faster than the mutant radius, and growth of both elements has essentially stopped by 6 weeks of age in mutants (Fig. 3D, solid blue bars versus solid green bar). While the compound mutant radius grows slower than controls throughout development, the decrease in growth rate over time is comparable to controls (Fig. 3D, solid green bar versus open green bar).

Bone mineral quality is not affected in Hox11 compound mutants

Bone quality of both Hox11 compound mutants and controls was analyzed by µCT analyses at 0, 2, 4, 6 and 8 weeks of age. No differences in any of the parameters measured, including cortical area and thickness, inner and outer perimeter, and tissue mineral density were observed in the humerus of Hox11 mutants (Fig. 2B). In contrast, cross-sectional views of the radius and ulna through the mid-diaphysis of control and Hox11 mutants at 8 weeks of age
highlight morphological differences in these animals. Note the bowing phenotype leads to a larger distance between the radius and ulna in compound mutants compared to controls (Fig. 4A,B). Compound mutant bones show measured increases in cortical area, cortical thickness, and bone perimeter compared to controls (Fig. 4C,D). No differences in tissue mineral density are observed between control and Hox11 compound mutants demonstrating that, despite differences in bone thickness and size, by this measure bone quality appears to be comparable (Fig. 4C,D).

**Appositional bone growth is not coordinated in Hox11 compound mutant radius and ulna**

To investigate appositional growth patterns, we utilized dynamic histomorphometry in the radius and ulna of control and compound mutants. New mineral deposition was analyzed by injection of dye at 2 weeks (xylenol orange label), 3 weeks (calcein label), and 4 weeks (alizarin complexone label) of age. Animals were assessed two days after the last injection. Growth patterns of control and compound mutants were analyzed at the mid-diaphysis where peak curvature is observed in mutant radii. Both xylenol orange (2 weeks) and alizarin complexone (4 weeks) are detected in the red channel, and new bone formed at these two time points can be distinguished by the presence of a green calcine label between the two labels. Rapid growth rates and high bone turnover make quantification of appositional growth rates unfeasible at these time points, however, qualitative differences in overall growth patterns between Hox11 compound mutants and controls can be assessed.

Within the cortical bone of wild-type controls, there are relatively large distances between labels indicating significant amounts of growth between time points (Fig. 5A-C). Additionally, the distance between labels is not uniform, highlighting the dynamic nature of postnatal skeletal growth. The 3-week label (green) is present as a mostly uninterrupted layer throughout both zeugopod bones, the 4-week label (surface red layer) is observed uniformly on the endosteum of both bones in the control, while the 2-week label (cortical red label) has been largely remodeled away by the time point of analyses (Fig. 5A-C). Appositional growth in control animals is observed only on the posterior-distal periosteal surface of the radius and the anterior periosteal surface of the ulna corresponding to ridges that are being patterned on the bone surface (Fig. 5A-C, blue arrows).
In contrast to control animals, appositional growth in \textit{Hox11} compound mutants is consistently unidirectional in both the radius and ulna. Posterior growth is observed endosteally (Fig. 5D-F, white arrowheads) and periosteally (Fig. 5D-F, blue arrows) in both the radius and ulna. This strong unidirectional growth pattern is established by 2 weeks of age and continues through 4 weeks as demonstrated by incorporation and retention of all three labels (Fig. 5D-F). Minimal incorporation of label on the anterior surface of the compound mutant radius and ulna indicates a lack of new bone formation or high levels of remodeling on this surface. Taken together, the data demonstrate that \textit{Hox11} mutants enact active bone growth in opposition to the direction of anterior radial bowing, consistent with correctional growth.

\textbf{Hox11 compound mutant growth plates are morphologically abnormal and chondrocytes are prematurely depleted}

Growth plate morphology was analyzed at 0, 2, 4, 6, and 8 weeks of age in controls and compound mutants to investigate whether premature depletion of chondrocytes contributes to the reduction in bone length in mutants. The growth plate length of the mutant radius and ulna is significantly shorter than controls at newborn stages despite the absence of significant differences in bone length at this stage, and the growth plate remains shorter throughout postnatal development (Fig. 6A,B). By 6 weeks of age and beyond, differences in growth plate length are no longer statistically significant, likely due to depletion of growth plate chondrocytes in both controls and mutants by these stages (Fig. 6A,B). O-V)

All three zones of the growth plate: the reserve zone, proliferative zone, and hypertrophic zone, are established in the compound mutant, however, cellular organization of the growth plate is disorganized compared to controls. This is noted particularly in the PZ of the compound mutants. PZ chondrocytes are visualized histologically in clusters in the mutant animals, in contrast to the normal vertical columns of flattened chondrocytes seen in controls (Fig. 6D, PZ vs Fig. 6C, PZ and Fig. 6F, PZ vs Fig. 6E, PZ). While matrix secreted by the PZ chondrocytes is not uniformly organized vertically in \textit{Hox11} compound mutants, there are no apparent differences in the amount of Safranin-O stained matrix produced compared to controls (Fig. 6D, PZ vs Fig. 6C, PZ and Fig. 6F, PZ vs Fig. 6E, PZ). Additionally, RZ chondrocytes are depleted more rapidly in mutants compared to controls; there are noticeably fewer cells in this zone by 2 weeks of age (Fig. 6H′ vs G′ and Fig. 6J′ vs I′).

To examine the cause of the premature shortening of the growth plate in \textit{Hox11} compound mutants, we examined proliferation rates in the RZ and PZ of the growth plate using BrdU labeling analysis. Since the number of RZ chondrocytes is noticeably reduced by 2 weeks of age in mutants, we focused our analysis to 0, 1, and 2 weeks of age. Proliferation in the mutant radius and ulna is higher in both the RZ and PZ than in controls, reaching statistical significance at 1 week in the ulna and 2 weeks in the radius.
DISCUSSION

Herein, we show that Hox11 genes continue to be expressed and function during postnatal skeletal development. While preserving a single functional allele of Hoxa11 or Hoxd11 is sufficient for normal embryonic bone development, the radius and ulna of Hox11 compound mutant animals develop abnormal skeletal morphology postnatally compared to controls. The progressive anterior bowing of the mutant radius does not appear to be due to inferior bone quality or mis-directed appositional growth. In fact, both the compound mutant radius and ulna exhibit appositional growth consistent with compensatory growth. We demonstrate that the anterior radius bowing phenotype is likely to be the result of disproportionate longitudinal growth of the radius and ulna, with the ulna lagging in growth rate compared to the radius. Uncoordinated growth leads to bowing of the faster-growing element, the radius. These data suggest that Hox11 genes function to coordinate the longitudinal growth of the distal growth plate of the radius and ulna.

Why the radius grows faster than the ulna in the Hox11 compound mutant is unclear. The genotype of the Hox11 compound mutant alone cannot explain the loss of coordinated growth of the radius and ulna since the radial bowing phenotype occurs to a similar extent in both compound mutant genotypes; Hox11Aadd or Hox11aaDd (Davis et al., 1995). Embryonically, a single Hoxa11 or Hoxd11 allele is sufficient to support normal...
forelimb development. Therefore, both elements of the zeugopod clearly require *Hox11* function for proper patterning during development. These observations highlight that *Hoxa11* and *Hoxd11* genes perform similar and highly overlapping functions during embryonic development and in regulating postnatal growth. *Hoxa11* and *Hoxd11* are strongly expressed in the mesenchymal

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**Fig. 4.** µCT analysis of bone morphology and quality. (A,B) Cross-sectional view of µCT scans at 8 weeks of age for control (A) and *Hox11* compound mutant (B) forelimb zeugopod. (C,D) Cortical area and thickness, inner and outer perimeter, and tissue mineral density of the radius (C) and ulna (D) of control (light grey) and compound mutant (dark grey) animals were measured at 0, 2, 4, 6 and 8 weeks of age. *P*<0.05; error bars depict standard deviation.
tissue surrounding the distal ends of the radius and ulna embryonically and postnatally. One hypothesis to explain why the ulna is more severely affected than the radius during postnatal growth is that there is a differential requirement for Hox11 function in the ulna compared to the radius. Future studies utilizing conditional alleles of Hox11 for complete loss of Hox function in postnatal development will further clarify the function of Hox11 in regulating longitudinal skeletal growth.

During longitudinal skeletal growth, there is tight regulation of proliferation and chondrocyte maturation within the growth plate. The majority of proliferation occurs within the columns of PZ chondrocytes, however, RZ chondrocytes also undergo proliferation, to add new cells to the PZ (Farnum and Wilsman, 1993; Abad et al., 2002; Ballock and O’Keefe, 2003; Beier, 2005). Through postnatal development, there is a reduction in proliferation within the growth plate and a decrease in the height of the growth plate, a process termed growth plate senescence (Nilsson and Baron, 2004). Senescence is a complex process; chondrocytes within the growth plate are only capable of a finite number of cell divisions and the balance of chondrocyte proliferation and timing of maturation determines bone length (Baron et al., 1994; Gafni and Baron, 2000; Nilsson and Baron, 2004; Schrier et al., 2006). We show that there is a loss of proliferative regulation within the growth plate and premature senescence in Hox11 compound mutants. Abnormally high levels of proliferation in both RZ and PZ chondrocytes are measured in the compound mutant ulna and radius, and longitudinal growth arrests earlier in these mutants. These observations suggest that chondrocyte maturation within the growth plate is dysregulated with loss of Hox11 function.

We, and others, have demonstrated that Hox11 gene expression is restricted to the perichondrium and RZ chondrocytes and that Hox11 genes are not expressed within the PZ and HZ chondrocytes.

Fig. 5. Appositional bone growth examined by dynamic histomorphometry. Wild-type control (A-C) and Hox11 compound mutant (D-F) animals were injected with xylenol orange (red), calcein (green), and alizarin complexone (red) at 2 weeks, 3 weeks and 4 weeks of age respectively. Representative bright field (A,D) and fluorescent (B,E) cross-sectional images of the mid-diaphysis were overlaid (C,F) to examine appositional growth patterns. Areas of appositional growth of the periosteal (blue arrows) and endosteal (white arrowheads) bone surface are highlighted. Scale bar 100 µm.
themselves (Boulet and Capecchi, 2004; Swinehart et al., 2013). Since Hox genes are transcription factors, this raises the possibility that Hox functions to maintain an undifferentiated state within the RZ chondrocyte progenitor pool and regulates when RZ chondrocytes enter the PZ and begin to differentiate. Loss of Hox within RZ chondrocytes may result in a loss of progenitor identity, and thus, premature differentiation and depletion of the chondrocyte progenitor pool. This hypothesis is consistent with the phenotypes observed in embryonic Hox11 loss of function limbs. Hoxa11−/−; Hoxd11−/− mutant limbs are severely shortened, growth plates do

Fig. 6. The distal growth plate of Hox11 compound mutant forelimb zeugopod bones undergoes premature senescence. (A-B) Total growth plate length of the radius (A) and ulna (B) from control (light grey) and compound mutant (dark grey) animals was measured from Safranin-O/Fast Green stained sections. Representative images of Safranin-O/Fast Green/Hematoxylin stained distal forelimb growth plates at 0 week (C−F), 2 week (G−J), 4 week (K−N), 6 week (O−R) and 8 week (S−V) old controls and compound mutants. Black brackets demarcate zones of the growth plate at 0, 2, and 4 weeks (C−J). RZ, reserve zone; PZ, proliferative zone; HZ, hypertrophic zone. *P<0.05; error bars depict standard deviation. Scale bar 200 µm.
not form, and chondrocyte maturation is perturbed (Boulet and Capecchi, 2004). Additionally, proliferation analysis in the Hox11 mutant radius and ulna show proliferation throughout the cartilage anlage in contrast to control limbs where proliferation is restricted to the distal growing ends. Similar to the postnatal limb, Hox11 genes are expressed at the distal end of the growing cartilage anlage.

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**Fig. 7. Proliferation defects are observed in the distal growth plate of Hox11 compound mutant forelimb zeugopod bones.** Proliferative index (PI) was calculated from total BrdU-positive nuclei relative to total nuclei in the reserve and proliferative zone at 0, 1 and 2 week in the radius (A) and ulna (B) of control (light grey) and compound mutant (dark grey) animals. Representative images of BrdU staining in the distal growth plate of the radius (C) and ulna (D) at E18.5, 1 week, and 2 weeks of control and Hox11 compound mutant. White brackets demarcate zones of the growth plate (C-D). RZ, reserve zone; PZ, proliferative zone; HZ, hypertrophic zone. *P<0.05; error bars depict standard deviation. Scale bar 200 µm.
embryonically; suggesting that Hox11 contributes to maintaining progenitor identity and regulating the rate of proliferation and differentiation of chondrocytes in both the embryonic and adult context.

Growth plate chondrocytes undergo a well-characterized differentiation program that is common to all long bones, however, the rate of growth between growth plates of different bones vary substantially (Wilsman et al., 1996a,b). Neither systemic regulation nor local feedback loops can easily account for these differences since these factors are present in all growth plates. Our current data is consistent with a model whereby the differential growth rates across the skeleton are controlled, at least in part, by the Hox proteins expressed in each region. It is well documented that during development Hox9-13 paralogs function in a highly region-specific fashion to pattern the appendages. We demonstrate that Hox11 function remains region-specific during postnatal development and regulates long bone growth through controlling chondrocyte maturation within the growth plate.

MATERIALS AND METHODS

Animals

Male and female mice heterozygous for both the Hoxa11 and Hoxd11 null alleles and the Hoxa11eGFP knock-in allele were previously described (Wellik and Capecchi, 2003; Nelson et al., 2008). All procedures followed protocols reviewed and approved by the University of Michigan’s Committee on Use and Care of Animals.

qPCR analysis

Specimens were dissected in PBS on ice. Forelimbs/forelimb buds were removed and soft tissues were dissected away from the radius and ulna. The whole embryonic day (E) 12.5 limb bud or whole radius and ulna at E18.5, 1 week, 2 weeks and 4 weeks were collected into Trizol. Adult lung tissue was collected into Trizol as a control. qPCR for Hox11 genes was performed with the following primer sets: Hoxa11F − CTTTTTCAAATGCAGAAT-GT, Hoxa11R − AGGCCCTGACCCATTGAAAT, Hoxd11F − AGTGACCACGGATACGCCGAG, Hoxd11R − ACACCAAGTACCCAGATCG.

Delta Ct values were calculated for each primer set relative to GAPDH at each time point assessed and normalized to expression in the adult lung. Stained skeleton were cleared successively in 20% glycerol:1% KOH, 50% glycerol:1% KOH and 100% glycerol. Forelimbs were removed and imaged on a Leica MZ125 stereo microscope.

To visualize growth plate morphology, cryosections were stained with Safranin-O/Fast Green/Hematoxylin as previously described (Thompson et al., 2002). Images were captured on an Olympus BX-51 upright light microscope with an Olympus DP70 camera. Measurements of the total growth plate length were performed using ImageJ software (Schneider et al., 2012).

Micro-computed tomography (µCT) analysis

Samples were scanned using an eXplore Locus SP microCT system (GE Healthcare). All specimens were scanned in water using the following parameters: voltage 80 kVp; current 80 µA; exposure time 1600 ms; voxel size in the reconstructed image 18 µm, isotropic. The data were processed and analyzed using MicroView (v 2.1.2 Advanced Bone Application; GE Healthcare Preclinical Imaging). First, the image was reoriented so that the anterior-posterior and longitudinal axes were aligned with the principal image axes. Next, the bone was manually segmented starting with a frame at the center of the bone and extending 72 µm on either side to identify a 144 µm region of interest (ROI). The tissue mineral density (TMD), cortical area, cortical thickness, and inner and outer perimeters were calculated. The total length of the radius, ulna and humerus was measured along the central curvature of the bone. A minimum of five animals were analyzed at each age.

Bone histomorphometry

Five animals each of control and Hox11 compound mutant were injected with xylene orange (90 mg/kg) (Sigma), calcine (15 mg/kg) (Sigma), and alizarin complexone (30 mg/kg) (Sigma) in PBS at 2 weeks, 3 weeks and 4 weeks of age respectively. Animals were sacrificed two days following the final injection and limbs were collected. Un-decalcified bones were processed and embedded into plastic as previously published (Smith et al., 2013) 200 µm thick sections were collected, mounted to plastic slides and polished to approximately 30 µm thickness. Images were captured at 20X magnification using a Zeiss Axiosvert 200M inverted microscope equipped with Apotome imaging system.

BrdU incorporation

Timed pregnant females, 1 week, and 2 week pups were injected intraperitoneally with bromodeoxyuridine (100 mg/kg)/fluorodeoxyuridine (12 mg/kg) (Sigma) in DPBS. Pregnant females were sacrificed 2 h after injection and 1 and 2 week pups were sacrificed 4 h after injection. Specimens were dissected in PBS on ice. Forelimbs were collected and the soft tissues were removed. Limbs were fixed for 3 days in 4% paraformaldehyde in PBS at 4°C, decalcified in 14% EDTA for one week prior to embedding into OCT media. Newborn limbs did not require decalcification and were embedded in OCT immediately after fixation. Cryosections were collected at 16 µm through the radius/ulna.

Endogenous Hoxa11eGFP expression at E18.5 was visualized without the use of an antibody, however the decalcification process results in high auto-fluorescence in adult tissues and an antibody against GFP was required to visualize expression at 2 weeks. Immunohistochemical staining was performed by blocking with donkey serum and incubation with primary antibody against GFP (Invitrogen, A-11122, 1:200) overnight at 4°C. Secondary antibody was incubated at room temperature: donkey anti-rabbit Alexa Fluor488 (Invitrogen, A21206, 1:1000). Section in situ hybridization was performed using 35S-labeled riboprobes using standard techniques (Schipani et al., 1997). Hoxd11 in situ probes were previously described (Yallowitz et al., 2011).

For skeletal preparations, newborn animals were skinned and fixed in 95% ethanol. Fixed skeletons were stained in Alcian Blue (76% ethanol:20% acetic acid) at 37°C for 48 h, rinsed in 95% ethanol, treated with 1% KOH for 4-5 h and stained with Alizarin Red in 2% KOH for 1 h.
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