**INTRODUCTION**

Pbx1 is a TALE (three amino acid loop extension) class homeodomain protein originally identified as the product of an oncogene in a subset of childhood leukemias (Nourse et al., 1990; Kamps et al., 1990). Subsequent studies have shown that Pbx1 is a homolog of *Drosophila* extradenticle (EXD) and a component of various protein complexes implicated in developmental gene expression (reviewed by Mann and Chan, 1996). The transcriptional activity of Pbx1 is regulated in part by nuclear import mediated by dimerization with other TALE class homedomain proteins such as Meis/Prep1 (Berthelsen et al., 1999). The latter are evolutionarily related to but distinct from Pbx proteins (Burglin, 1997; Chen et al., 1997; Moscow et al., 1995), with which they form stable, nuclear DNA-binding complexes (Bischof et al., 1998; Chang et al., 1997; Knoepfler et al., 1997; Berthelsen et al., 1998a; Berthelsen et al., 1999). Biochemical analyses suggest that heterodimers of Pbx and Meis-related proteins contribute to the regulation of several genes (Berthelsen et al., 1998; Berthelsen et al., 1999; Bischof et al., 1998; Swift et al., 1998) in different tissues.

As a TALE heterodimer, Pbx displays an ability to simultaneously interact and bind DNA with Hox proteins thereby forming trimeric complexes on appropriate DNA sites (Berthelsen et al., 1998; Jacobs et al., 1999; Schnabel et al., 2000). Furthermore, two essential Hoxb1 hindbrain enhancers contain DNA binding sites that support the in vitro assembly of trimeric Hox-TALE complexes and are necessary for hindbrain enhancer activity in vivo (Jacobs et al., 1999; Ferretti et al., 2000). Numerous studies have demonstrated that interactions with Pbx1 result in enhanced Hox DNA binding affinities and specificities in vitro (Chang et al., 1994; Chang et al., 1995; Chang et al., 1996; Knoepfler and Kamps, 1995; Lu et al., 1995; Peers et al., 1995; Peltenburg and Murre, 1996; Phelan et al., 1995; Shen et al., 1996; van Dijk and Murre, 1994). These interactions are confined to a subset of Hox and other homeodomain proteins that contain characteristic tryptophan-bearing dimerization motifs. Genetic analyses in

**SUMMARY**

Pbx1 and a subset of homeodomain proteins collaboratively bind DNA as higher-order molecular complexes with unknown consequences for mammalian development. Pbx1 contributions were investigated through characterization of Pbx1-deficient mice. Pbx1 mutants died at embryonic day 15/16 with severe hypoplasia or aplasia of multiple organs and widespread patterning defects of the axial and appendicular skeleton. An obligatory role for Pbx1 in limb axis patterning was apparent from malformations of proximal skeletal elements, but distal structures were unaffected. In addition to multiple rib and vertebral malformations, neural crest cell-derived skeletal structures of the second branchial arch were morphologically transformed into elements reminiscent of first arch-derived cartilages. Although the skeletal malformations did not phenocopy single or compound Hox gene defects, they were restricted to domains specified by Hox proteins bearing Pbx dimerization motifs and unaccompanied by alterations in Hox gene expression. In affected domains of limbs and ribs, chondrocyte proliferation was markedly diminished and there was a notable increase of hypertrophic chondrocytes, accompanied by premature ossification of bone. The pattern of expression of genes known to regulate chondrocyte differentiation was not perturbed in Pbx1-deficient cartilage at early days of embryonic skeletogenesis, however precocious expression of *Coll1a1*, a marker of bone formation, was found. These studies demonstrate a role for Pbx1 in multiple developmental programs and reveal a novel function in co-ordinating the extent and/or timing of proliferation with terminal differentiation. This impacts on the rate of endochondral ossification and bone formation and suggests a mechanistic basis for most of the observed skeletal malformations.

Key words: Pbx1, Skeleton, Limb, Branchial arch homeosis, Hox, Chondrocyte proliferation, Mouse
both mice and Drosophila provide in vivo support that Pbx and EXD (Peifer and Weischaus, 1990; Rausklob et al., 1993) function in concert with Hox proteins during development through response elements containing their cognate DNA binding sites (Chan et al., 1994; Maconochie et al., 1997; Popperl et al., 1995). Thus, Pbx proteins appear to play diverse roles as components of various protein complexes to orchestrate transcriptional programs in multiple tissues.

Although acquired Pbx1 somatic mutations are associated with hematopoietic cancer (Hunger, 1996), the lack of Pbx1 germline mutants has compromised a refined analysis of its in vivo contributions to mammalian development. In this report we have generated and characterized Pbx1-deficient mice. Pbx1 is an essential gene, whose loss results in late gestational lethality, accompanied by severe hypoplasia or aplasia of multiple organs. A necessary role in patterning is apparent from widespread defects of the axial and appendicular skeleton, including morphological transformation of skeletal elements of the second branchial arch. Furthermore, absence of Pbx1 causes abnormalities in chondrocyte proliferation and differentiation, which result in precocious ossification and bone formation. These studies reveal a novel function of Pbx1 in coordinating the extent of chondrocyte proliferation and terminal differentiation, suggesting a mechanistic basis for most of the observed skeletal malformations.

MATERIALS AND METHODS

Targeted disruption of the Pbx1 gene and generation of Pbx1 knockout mice

The Pbx1 gene was mutated by insertion of a PGK-neo cassette (from the pNT vector) into the unique Nhel site of exon 3, the largest 5’ exon containing a non-unit number of codons. An 8.5 kb segment of genomic DNA spanning the disrupted Pbx1 exon 3 was then cloned into the targeting vector containing the Herpes Simplex Virus thymidine kinase (HSV-tk) cassette (Fig. 1). The targeting construct was linearized by NotI digestion and then electroporated into ES cells (TL1 line; Labosky et al., 1997). Following positive/negative selection in G418 and gancyclovir, homologous recombinant clones were identified by Southern blot analysis using three different enzyme and probe combinations (5’ and 3’ external probes and a neo-specific internal probe). Out of 95 informative clones, 8 yielded restriction digest patterns diagnostic for homologous recombination. Euploid clones were micro-injected into C57BL/6j host blastocysts. Chimeric male mice from two independently derived ES clones (38 and 176) passed the targeted Pbx1 allele through the germline. Phenotypes were analyzed in embryos derived from the third backcross generation on a C57BL/6 background and on a fully inbred 129/SvTer background. The homozygous (~~/~) phenotype was identical in mice derived from either of two independently targeted ES clones (38 and 176). Genotype analysis was performed on DNA extracted from tail biopsies of adult mice or from yolk sacs of embryonal conceptuses (E10-19) dissected free of maternal tissues. Following digestion with SspI, DNAs were subjected to Southern blot analysis using the Pbx1 3’ external probe.

Western blot analysis

Whole embryos at E16 were lysed in 2x SDS sample buffer following homogenization using established procedures (Jacobs et al., 1999). Proteins were subjected to SDS-polyacrylamide gel electrophoresis and immobilized on nitrocellulose filters following electrophoretic transfer. Pbx1 proteins were detected by western blot analysis using a monoclonal antibody (aPbx1b) specific for the Pbx1b isoform (the most abundant of two translation products that arise from differential splicing of the Pbx1 transcript) or an anti-Pbx1 rabbit antiserum raised against the amino terminus of Pbx1 (P-20; Santa Cruz Biotechnology).

Histology and immunohistochemistry

For histological analysis, embryos were fixed in formalin and embedded in paraffin for sectioning using standard procedures. Sections of 5 μm thickness were stained with Hematoxylin and Eosin (H and E) and von Kossa, mounted in DPX and photographed. Immunohistochemistry was performed on dewaxed, paraffin sections following microwave antigen retrieval (when necessary for select antibodies). Primary antibodies consisted of: Pbx1b-specific (aPbx1b) and pan Meis-specific monoclonal antibodies (Ab) (Jacobs et al., 1999); polyclonal anti-collagen X (Kwan et al., 1997) (generously provided by Olena Jacenko); monoclonal anti-PCNA and polyclonal antisera against collagen II, MMP-2, MMP-3 and MMP-9 (all purchased from Santa Cruz Biotechnology); monoclonal anti-BrdU (purchased from Neo Markers).

Analysis of BrdU incorporation

Pregnant mice were injected intravenously with 50 μg bromodeoxyuridine (BrdU) per gram of body weight 3 hours before sacrifice. Embryos were treated as above and embedded in paraffin before transverse sectioning. BrdU was detected by immunohistochemistry as described previously (Nowakowski et al., 1989) and sections were counterstained lightly with H and E. All BrdU-positive (dark brown) and -negative (blue) nuclei were counted over various rib sections. At least 15 different sections were counted for each of two wild-type and two Pbx1+/− littermate embryos at E12.5, 13.5 and 14.5.

Skeletal preparations

Differential staining of cartilage and bone in whole mouse embryos (E16) was visualized using Alcian Blue and Alizarin Red S (Mcleod 1980; Depew et al., 1999).

Whole-mount in situ hybridizations

Whole-mount in situ hybridizations were performed on embryos at E9.5, E10.5 and E11.5 as previously described (Wilkinson and Green, 1990). Hybridization probes for the clustered Hox genes (a3, b1, b9, d3, d9 and d11) and branchial arch markers (Lhx6, dHAND, Gsc, Hoxa2) have been described in previous studies and their compositions are available upon request. All probes were labeled with digoxigenin, using standard procedures.

Section in situ hybridizations

In situ hybridizations were performed both on paraffin and frozen sections, of embryos at E12.5, E13.5 and E15.5, using digoxigenin and 35S-labeled riboprobes, as previously described (Wilkinson, 1992). Single stranded sense and antisense riboprobes were generated which were specific for Indian hedgehog (Ihh; Bitgood and McMahon, 1995), Sox9 (Wright et al., 1995; Ng et al., 1997), Fgfr3 (Deng et al., 1996), parathyroid hormone-related peptide and its receptor, PTHrP and PTHrPR (Lanske et al., 1996), Col2al type IIa form, Col10al and Col11al (Cheah et al., 1995). In situ hybridizations were carried out on adjacent sections to facilitate comparison of the expression patterns.

RESULTS

Pbx1 is essential for fetal development

A null allele of the endogenous mouse Pbx1 gene was created by insertion of a neomycin resistance gene into exon 3 through homologous recombination in ES cells (Fig. 1). The
Fig. 1. Targeted inactivation of \textit{Pbx1} and gross morphology of wild-type and mutant embryos. (A) Schematic representation of the mouse \textit{Pbx1} cDNA, targeting vector and mutated allele following homologous recombination. In the \textit{Pbx1} cDNA, sequences derived from exon 3 and the homeodomain (HD) are shown as black and gray boxes, respectively. Approximately 20 kb of the \textit{Pbx1} locus flanking exon 3 (black box) are depicted along with mapped restriction sites. The targeting construct carries a PGK-neo cassette, inserted into the unique \textit{NheI} site of \textit{Pbx1} exon 3, and the HSV-tk gene, shown as solid white boxes. The transcriptional orientations of the \textit{Pbx1} arms of homology are opposite to that of the PGK-neo cassette. The 5' and 3' external probes used for Southern blot analyses are shown as solid black boxes below the mutated allele.

Restriction enzyme sites: E, EcoRI; N, NheI; S, SspI; X, XhoI. Xmal and NotI sites are in the targeting vector; the SspI site (S) was introduced to facilitate diagnostic analysis. (B,C) Southern blot analysis of \textit{Pbx1} alleles. DNA from cell lines (B) or mouse tissues (C) was analyzed with probes and enzymes indicated beneath the panels. Wild-type (16 and 6.5 kb) and mutant (7.2 and 5.5 kb) \textit{Pbx1} alleles for \textit{Xmal} and \textit{SspI} digests, respectively, are indicated to the right of each panel. TL, ES cell line used for \textit{Pbx1} gene targeting; clones 38 and 176, targeted ES cells that passed the \textit{Pbx1} mutation through the germ line; MEFs, mouse embryonic fibroblasts. (D) Western blot analysis of \textit{Pbx1b} expression. Protein extracts of E16 embryos were subjected to western blot analysis using a monoclonal antibody specific for the \textit{Pbx1b} isoform. Genotypes determined by Southern blotting are listed at the top. Right lane contains in vitro translated \textit{Pbx1b}. Migrations and sizes (kDa) of molecular mass standards are indicated to the right. (E,F) Gross morphology of wild-type and \textit{Pbx1-/-} embryos at E13.5 (E) and E16 (F). Mutant embryos display massive subcutaneous edema (yellow arrowhead), pallor, slender thorax and abdomen, hypoplastic pinna (red arrowhead) and atypical hunched posture with abnormal orientation of both hind- and forelimbs (black arrowheads).

Expected wild-type and mutated \textit{Pbx1} alleles were observed by Southern blot analysis of DNA extracted from targeted ES cell lines and mouse tissues (Fig. 1B,C). Western blot analysis of embryos at E16 showed that homozygous mutant embryos expressed neither full-length \textit{Pbx1b} proteins (Fig. 1D) nor truncated amino-terminal fragments of \textit{Pbx1} (data not shown).

\textit{Pbx1}^{+/-} mice expressed half the normal level of \textit{Pbx1b} proteins (Fig. 1D) but were viable, fertile and displayed no apparent abnormalities other than a decreased size (30% by weight prior to 8 weeks of age). Intercross matings of \textit{Pbx1}^{+/-} mice yielded no viable \textit{Pbx1}^{-/-} pups with the exception of one stillborn neonate out of 77 \textit{F2} offspring (Table 1). Binomial proportion analysis showed no deviations from a 2:1 heterozygote:wild-type ratio (Table 1), suggesting that \textit{Pbx1} null embryos died during gestation.

\textit{Pbx1}^{-/-} embryos from timed intercross matings were analyzed to determine the specific stage of embryonic lethality (Table 1) and to study the lethal phenotype. Prior to E12.5, \textit{Pbx1}^{-/-} embryos could not be distinguished morphologically from wild-type or heterozygous embryos. Subsequently, mutant embryos showed massive and progressive subcutaneous edema (Fig. 1E). They also exhibited generalized pallor, diminished vasculization, slender thorax and abdomen, hunched posture, abnormal orientation of both the fore and hind limbs, and hypoplastic ear pinnae (Fig. 1E,F). Furthermore, in \textit{Pbx1}^{-/-} embryos many internal organs were severely hypoplastic (e.g. lungs, liver, stomach, gut, kidneys and pancreas), ectopic (thymus, kidneys) or aplastic (spleen) (Table 2 and data not shown). Nonetheless, until E14.5 all \textit{Pbx1}^{-/-} embryos were alive, as evidenced by heart beat and pulsation of arterial blood through the umbilical artery, and were obtained in the expected Mendelian ratio (Table 1). Thereafter, an increasing number of \textit{Pbx1}^{-/-} embryos were dead, beginning at E15.5, and none were alive at E17.5 (Table 1). These data established that \textit{Pbx1} is an essential gene whose loss results in late gestational lethality. Here we focus on the phenotypic consequences of the loss of \textit{Pbx1} in the developing skeleton.

\begin{table}[h]
\centering
\begin{tabular}{lllllll}
\hline
\textbf{Stage} & Total & \textbf{+/-} & \textbf{+/-} & \textbf{Necrotic*} & \textbf{Alive} & \textbf{Dead} \\
\hline
E11.5 & 37 & 12 & 15 & 10 & 0 & 0 \\
E12.5 & 29 & 8 & 14 & 7 & 0 & 0 \\
E13.5 & 33 & 9 & 14 & 10 & 0 & 0 \\
E14.5 & 56 & 8 & 39 & 9 & 0 & 0 \\
E15.5 & 50 & 11 & 22 & 12 & 5 & 0 \\
E16.5 & 50 & 11 & 22 & 12 & 5 & 0 \\
E17.5 & 28 & 6 & 11 & 0 & 5 & 6 \\
E18.5 & 29 & 5 & 15 & 0 & 4 & 5 \\
F2 Pups & 77 & 26 & 50 & 0 & 1 & 0 \\
\hline
\end{tabular}
\caption{Genotypes of embryos obtained from intercrosses of \textit{Pbx1}^{+/-} mice.}
\end{table}

*Genotyping was not possible.
Pbx1 expression and skeletal structures in wild-type and mutant embryos.

(A-D) Immunohistochemical analysis of Pbx1b localization visualized with DAB (brown staining) on tissues counter-stained with Hematoxylin. Diffuse nuclear Pbx1b expression is present in the epidermis and mesenchymal tissues of the embryo at E13.5 (A), most intensely in mesenchyme that is condensing to form rib (black arrowheads) and vertebral (red arrowheads) cartilage (B). Abundant Pbx1b expression in proliferating rib chondrocytes at E14.5 (C), which is nuclear (C, inset), is reduced to undetectable levels in hypertrophic chondrocytes at E16 when ossification is imminent (D). BA2, second branchial arch; vert, vertebrae. (E,F) Differential bone and cartilage staining of skeletal elements. E16 embryos were stained with Alcian Blue and Alizarin Red to visualize cartilage and bone, respectively, in the forming skeletal structures. Lateral views demonstrate agenesis of the ventral ribs, hypoplasia of the sternum and clavicles, together with rib malformations and fusions (red arrowhead) in the Pbx1<sup>−/−</sup> embryo (E, right). There is also thinning, compressions (black arrows) and fusions (red-tipped arrows) of the vertebral bodies and neural arches in the Pbx1<sup>−/−</sup> embryo (F, bottom). The stylohyoid process and hyoid lesser horns (green arrows) are malformed, as are the structures of the occipital arch. Costal cartilage, Cc; clavicle, cv; exoccipital, EO; otic capsule, OC; ossified rib, orb; supraoccipital arch, SOA; sternum, stn. Asterisks indicate malformed structures.

Pbx1<sup>−/−</sup> embryos display axial skeletal malformations that are not segmentally restricted

Previous studies have shown that Pbx1 is transcribed widely throughout the embryo (Roberts et al., 1995; Schnabel et al., 2001). Nuclear-localized Pbx1 protein was detected by immunohistochemistry in many immature epithelial and mesenchymal cells, particularly in condensing pre-cartilagenous mesenchyme (Fig. 2A,B). It was also highly expressed in the nuclei of proliferating chondrocytes at E14.5 (Fig. 2C), but progressively decreased with further chondrocyte maturation and was undetectable in hypertrophic chondrocytes at the onset of ossification (Fig. 2D). Consistent with this expression profile, Pbx1<sup>−/−</sup> embryos showed widespread abnormalities involving both the axial and the appendicular skeleton (Fig. 2E).

Within the axial skeleton, agenesis of the ventral portions of all 13 ribs was observed, together with thickening and fusions (arrowhead) of shortened ribs (Fig. 2E). The sternum was severely hypoplastic (Fig. 2E). The cervical and thoracic vertebrae were thinned, their arches were occasionally fused (black arrows), and the intervertebral spaces were reduced (Fig. 2E,F). These features were most severe in the C1, C2 and C3 cervical vertebrae. The expected numbers of vertebrae were observed and the vertebral and rib malformations showed some left-right asymmetries (data not shown). In addition to the vertebral alterations, the cranial skeletal elements derived from the occipital arch (exoccipital and supraoccipital), otic capsule and parachordal plate (basiooccipital and basisphenoid) were abnormal (arrowhead Figs 2F, 3C). Thus, Pbx1<sup>−/−</sup> mice displayed widespread axial skeletal malformations.

Anterior homeotic transformation of second branchial arch structures in Pbx1<sup>−/−</sup> embryos

Structures derived from the second branchial arch and first pharyngeal groove displayed striking morphological changes in Pbx1<sup>−/−</sup> mutants. The main source of ectomesenchyme in the second branchial arch, the rostral-most Hox-positive arch, is cranial crest-derived from rhombomere 4 (Couly et al., 1996; Kontges et al., 1996). Nuclear Pbx1 was detectable by immunohistochemistry in the ectomesenchyme and ectoderm of the second branchial arch and around the first pharyngeal groove at E11.5, while the ectomesenchyme of the mandibular and maxillary branches of the first branchial arch showed much lower nuclear immunoreactivity (Fig. 3A). Consistent with the...
**Fig. 3.** Transformation of second branchial arch cartilages into structures resembling mandibular first arch cartilages in Pbx1\(^{-/-}\) embryos as determined from anatomic, histological and molecular analyses.

(A) Immunohistochemical analysis shows extensive Pbx1b expression in mesenchyme of the second branchial arch and first pharyngeal groove of E11 and E13.5 wild-type embryos (transverse section). Auricular pinna, AP; first branchial arch, BA1; second branchial arch, BA2; Meckel’s cartilage, MC; mandibular arch, MdA; maxillary arch, MxA; optic cup, opc; otic capsule, OC; first pharyngeal groove, PG1; styloid process, SP; spinal cord, spc; tongue, Tg.

(B) Lateral views of wild-type (top) and mutant (bottom) E16 head skeleton. The lesser horns of the mutant are greatly elongated, resembling Meckel’s cartilage (or BA2 homologs of non-mammalian vertebrates). They extend from the hyoid body and end adjacent to the styloid process. The stapes is absent, and the styloid process is also dysmorphic, being shorter, thicker and having an ectopic flange (yellow arrowhead). Its shape resembles, in parts, a malleus. Proximocaudal mandibular arch structures are slightly dysmorphic, as exemplified by the caudal border of the malleus. There is a small, ectopic cartilaginous spicule near the malleus (green arrowhead).

(C) Inferior views of the wild-type (left) and mutant (right) head showing the branchial arch defects, in addition to malformations (red arrowhead) of the cranial base. Green and yellow arrowheads denote structures described in B.

(D) Morphologies of dissected hyoid and mandible cartilages. Asterisks indicate malformed structures. Abbreviations: atlas, at; dentary, dnt; exoccipital, eo; greater horn of hyoid, gh; hyoid body, hb; incus, in; lesser horn of hyoid, lh; transformed lesser horn of hyoid bone, lh*; malleus, ma; Meckel’s cartilage, MC; nasal capsule, nc; otic capsule, OC; supra capsular fissura, scf; styloid process, sp.

(E) Histology of H and E-stained transverse sections of wild-type and Pbx1\(^{-/-}\) embryos at E16. The transformed and elongated lesser horns of the hyoid bone are indicated by an asterisk. greater horn of hyoid, gh; lesser horn of hyoid, lh; transformed lesser horn of hyoid bone, lh*; Meckel’s cartilage, MC; oropharynx, o.

(F) Same as E, magnified 100×. The transformed lesser horn of the hyoid consists of tightly packed, proliferating chondrocytes. (G,H) Whole-mount in situ hybridization analysis of Lhx6 and dHAND expression in wild-type and Pbx1\(^{-/-}\) embryos at E10.5.
strong nuclear expression in the second branchial arch, \( Pbx1^{-/-} \)-mutants had a striking morphological alteration in the

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**Table 2. Phenotypic overlaps in \( Pbx1 \) and \( Hox \) knockout mice**

<table>
<thead>
<tr>
<th>( Pbx1 ) phenotype</th>
<th>( Hox^* ) null mutants</th>
<th>( Hox ) Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior transformation of BA2</td>
<td>( Hoxa2 )</td>
<td>Rijli et al., 1993; Gendron-Maguire et al., 1993</td>
</tr>
<tr>
<td>Cervical vertebral malformations</td>
<td>( Hoxa5b/5d3 )</td>
<td>Manley and Capecchi, 1997</td>
</tr>
<tr>
<td>Rib malformations</td>
<td>( Hoxa9/9b )</td>
<td>Chen and Capecchi, 1997</td>
</tr>
<tr>
<td>Forelimb skeletal malformations</td>
<td>( Hoxa9/9d )</td>
<td>Fromental-Ramain et al., 1996</td>
</tr>
<tr>
<td>Proximal hindlimb skeletal malformations</td>
<td>( Hoxa10 )</td>
<td>Favier et al., 1996</td>
</tr>
<tr>
<td>Cranial nerve defects†</td>
<td>( Hoxa1/1b1 )</td>
<td>Gavalas et al., 1998</td>
</tr>
<tr>
<td>Impaired hematopoiesis§</td>
<td>( Hoxa9/9c8 )</td>
<td>Lawrence et al., 1997; Shimamoto et al., 1999</td>
</tr>
<tr>
<td>Ectopic thymus¶</td>
<td>( Hox3 ) paralogs</td>
<td>Manley and Capecchi, 1998</td>
</tr>
<tr>
<td>Spleen agenesis**</td>
<td>( Hox1i )††</td>
<td>Roberts et al., 1994; Dear et al., 1995</td>
</tr>
<tr>
<td>Pancreas abnormal development and function‡‡</td>
<td>( Pdx1 )†‡</td>
<td>Jonsson et al., 1994; Offield et al., 1996</td>
</tr>
</tbody>
</table>

*All listed clustered and non-clustered \( Hox \) proteins bear \( Pbx1 \) dimerization motifs.
†Depew and Selleri, unpublished observations.
¶Manley, Selleri and Ciley, unpublished.
**Selleri et al., unpublished.
††Non-clustered \( Hox \) genes.
‡‡Kim et al., unpublished.
The malleus was slightly dysmorphic at its articulation with the incus and along its caudal border. The pinnae of the ears, which develop around the first pharyngeal groove, were hypoplastic and malformed (Fig. 1F). Therefore, loss of Pbx1 transformed second branchial arch cartilages into structures resembling splanchnocranial elements of the first branchial arch. Histologic analysis of BA2 at E16 (Fig. 3E,F) further showed an elongated structure, reminiscent of Meckel’s cartilage, consisting of small proliferating tightly packed chondrocytes, in place of the lesser horns of the hyoid.

Several molecular markers of branchial arch development were examined at E10.5 to determine their roles in transformation of branchial arch morphology associated with the loss of Pbx1. These consisted of genes normally expressed exclusively in BA1 (Lhx6), genes normally expressed in the mesenchyme of BA2 (Hoxa2), and genes with proximodistally restricted expression within the branchial arches (dHAND, Gsc). We detected no major changes in the expression of these genes (Fig. 3G,H and data not shown).

### Malformations of the proximal but not distal appendicular skeleton in Pbx1−/− mutants

A possible role for Pbx1 in patterning the vertebrate limbs was suggested by its restricted nuclear localization within the proximal domains of the limb bud, a feature shared with its Drosophila homolog exd (González-Crespo et al., 1998). In E11.5 wild-type embryos, Pbx1 protein was strongly expressed in the nuclei of mesenchymal cells of the proximal limb bud, but not in those of the distal bud (Fig. 4A). Meis proteins were detected in mesenchymal cell nuclei in a similar proximally restricted pattern in both wild-type and Pbx1−/− embryos (Fig. 4A). These data are consistent with the regulation of Pbx1 nuclear import by Meis-related TALE proteins (Berthelsen et al., 1999) in developing proximal but not distal limb buds.

By E14.5, skeletal structures of the proximal limbs and limb girdles, tissues that normally express nuclear Pbx1, were clearly abnormal in Pbx1−/− embryos, and by E16 the scapula and clavicle were malformed and the humerus was fused to the scapula (Fig. 4B). The proximal humerus was hypoplastic and misshapen, and the deltoid tuberosity was not conjoined. In contrast, distal forelimb elements and joints of the zeugopod and autopod appeared normal (Fig. 4B). In the hind limbs, the
pelvic girdle and proximal femur were malformed with a rudimentary ilium, ischium and pubis, and the os coxae-femur articulation appeared to be fused (Fig. 4C). As with the fore limb, distal elements and joints of the hind limb appeared normal (Fig. 4C). Thus, limb development in mammals is likely compartmentalized into Pbx1-dependent (proximal) and Pbx1-independent (distal) domains, as in arthropods (González-Crespo et al., 1998).

Absence of Hox and Meis expression perturbations in Pbx1−/− embryos

No differences were observed between wild-type and Pbx1−/− embryos in the spatial and temporal expression profiles of several Hox genes that normally contribute to the specification of structures displaying malformations in the Pbx1−/− mice (data not shown). These analyses examined Hoxa3 (cervical vertebrae, hyoid), Hoxb1 (hindbrain), Hoxb9 (sternum and ribs), Hoxd3 (cervical vertebrae), Hoxd9 (proximal fore limb), and Hoxd11 (distal limbs), at two different days (E9.5 and E11.5) of gestation. Furthermore, no perturbations of Meis expression or subcellular distribution were observed in the proximal limb buds (Fig. 4A) and other structures (not shown) of Pbx1−/− embryos. Thus, Meis nuclear import was not dependent on the presence of Pbx1 protein.

Accelerated progression of endochondral ossification in Pbx1−/− embryos

Histologic analysis was conducted on the developing skeleton in domains that displayed gross morphologic differences between wild-type and Pbx1−/− embryos. At E13.5, wild-type hyaline cartilage of the ribs contained aggregations of small, tightly packed chondrocytes (Fig. 5A,B). Focally, in the central portions of the ribs, Pbx1−/− chondrocytes displayed subtle features of more advanced maturation compared to wild type, as shown by the presence of a significant number of cells with markedly vacuolated cytoplasms (Fig. 5C,D). At E15.5, marked differences in chondrocyte maturation were readily apparent (Fig. 5E-H). Many more hypertrophic chondrocytes were observed in affected Pbx1−/− cartilage compared to wild type. Numerous chondrocytes were clearly undergoing autolysis, surrounded by extensive bone formation beneath the perichondrium (Fig. 5G,H). Representative data are shown for whole embryos that were entirely sectioned and stained. In addition, von Kossa staining of E15.5 Pbx1−/− rib cartilages showed the presence of extensive calcium deposits that were practically absent from wild-type cartilage, even in areas where chondrocytes had undergone hypertrophy (Fig. 5M,N), which is indicative of precocious mineralization and accelerated ossification of the mutant cartilage. Similar defects in chondrocyte differentiation were also seen in the proximal limbs (data not shown) but not in the distal limbs (Fig. 5I-L). Expansion of hypertrophic chondrocytes was not observed in the splanchnocranium of BA2 (Fig. 3E).

Diminished chondrocyte proliferation in Pbx1−/− embryos

Growth within cartilagenous elements is dependent primarily on proliferation of chondrocytes prior to chondrocyte hypertrophy. Therefore, chondrocyte proliferation was
Precocious bone formation in Pbx1−/− mutants

To gain insight into molecular changes that may underlie the abnormal chondrocyte differentiation associated with the loss of Pbx1, the expression of genes characteristic for different stages of chondrocyte differentiation and maturation (Wai et al., 1998; Olsen et al., 2000) was examined for alterations in pattern and/or onset. Genes examined included the matrix genes Col2a1 type IIA (Ng et al., 1993), Col10a1 and Colla1 (Cheah et al., 1995) and genes that regulate chondrogenesis, such as Fgfr3 and Sox9 (Deng et al., 1996; Colvin et al., 1996; Ng et al., 1997; Bi et al., 1999), Ihh (St-Jacques et al., 1999), PTHrP and PTHrPR (Karaplis et al., 1994; Lanske et al., 1996).

Transcripts for Col2a1 type IIA form (a marker of pre-chondrocytes and proliferating chondrocytes) were present in both wild-type and Pbx1−/− ribs at E12.5 and E13.5. Immunostaining showed that collagen II was expressed at E15.5 in both wild-type and Pbx1−/− ribs (data not shown). However, at E15.5, both Col2a1 and collagen II were substantially reduced in Pbx1−/− rib cartilage, while expression was maintained in wild-type cartilage (Fig. 6A compared with B). At E13.5, staining for collagen X, which is characteristic of hypertrophic chondrocytes, was present in a few cells in wild-type ribs, but weak and more intracellular. By contrast, at E13.5, Pbx1−/− ribs showed distinct presence of collagen X in the matrix surrounding the hypertrophic chondrocytes (data not shown). This difference between Pbx1−/− and wild type was
very prominent by E15.5 when collagen X was abundant in the extracellular matrix (and focal pericellular regions) of Pbx1−/− ribs (Fig. 6D), while staining was much less extensive in wild-type ribs that have only a few hypertrophic chondrocytes (Fig. 6C).

Rib cartilages were also evaluated for the presence of several matrix metalloproteinases normally expressed in chondrocytes (Ito et al., 1995; Nagase, 1998). At E13.5, MMP-2 (gelatinase A; Yu et al., 1998), MMP-9 (gelatinase B; Vu and Werb, 1998), and MMP-3 (stromelysin 1; Sternlicht et al., 1999) were present in both wild-type and Pbx1−/− cartilage. However, at E15.5 they were no longer detectable in Pbx1−/− cartilage (Fig. 6E,F and data not shown), providing further evidence that Pbx1−/− chondrocytes at E15.5 exhibited features of more mature cells.

No major changes in the overall patterns of expression of Fgf3 and Sox9 (markers of proliferating chondrocytes), Ihh (marker of prehypertrophic chondrocytes), PTHrP and PTHrPR (markers of maturing and prehypertrophic chondrocytes) were detected in rib cartilages at early stages of skeletogenesis (E12.5 and 13.5; Fig. 7A-J and data not shown).

At E15.5 there were fewer cells positive for Fgfr3, PTHrp, and Sox9, which is consistent with the strikingly reduced numbers of proliferating chondrocytes and the presence of many more hypertrophic chondrocytes that do not express these genes (Fig. 7G-L and data not shown). Ihh and PTHrPR were expressed appropriately in prehypertrophic chondrocytes in both wild-type and Pbx1−/− rib cartilage at the three stages examined. At E15.5, expression of these chondrogenic regulators was physiologically lost in Pbx1−/− hypertrophic chondrocytes of rib cartilage (Fig. 7C,D and data not shown), as expected, since most chondrocytes were undergoing accelerated autolysis and precocious mineralization (Fig. 5G,H,M,N).

In situ hybridization for Coll1a1, a marker for bone, showed significant expression in the perichondrium surrounding the ribs in Pbx1−/− embryos at E15.5, while no signal was present in wild-type perichondrium, suggesting that bone formation was precocious (Fig. 7M-P). Taken together, these studies demonstrate that lack of Pbx1 results in markedly diminished chondrocyte proliferation between E12.5 and E13.5 of mammalian endochondral skeletal development in non-cranial skeletal domains with grossly evident patterning defects. This
is associated with abnormal terminal differentiation (hypertrophy) and precocious bone formation by E15.5.

DISCUSSION

Mice lacking Pbx1 function have abnormalities of several organs, many of which are either severely hypoplastic, ectopic, or aplastic (Table 2). Here we have focused on the consequences of Pbx1 loss on skeletal development, including the morphogenesis of mesenchymal structures derived from the branchial arches and limb buds. These tissues can be segregated into Pbx1-dependent (caudal branchial arches and proximal limb buds) and Pbx1-independent domains (Fig. 8), demonstrating that Pbx1 is part of an ancient code, some of whose features have been conserved from Drosophila exd to higher vertebrates, for establishment of the body plan. However, our studies also reveal a previously uncharacterized role for Pbx1 in regulating the developmental programs of specialized cells in vertebrates, by temporally coordinating the extent of progenitor cell proliferation and commitment to terminal differentiation.

Essential role for Pbx1 in the refinement of multiple developmental programs

Pbx1−/− embryos die in utero and show diffuse patterning defects of the axial and proximal appendicular skeleton, as well as abnormalities in other organs. Some non-skeletal phenotypes, such as the spleen agenesis (Table 2), recapitulate phenotypes caused by the lack of ‘orphan’ Hox proteins, such as Hox 11, that bear Pbx dimerization motifs and heterodimerize with Pbx1 (Shen et al., 1996). The skeletal abnormalities, conversely, do not precisely recapitulate reported Hox null phenotypes (either single or compound; Capecchi, 1997) despite spatial overlap and morphological similarities. Their anatomic distributions, however, are associated with domains specified by Hox proteins containing trypotphan dimerization motifs essential for cooperative DNA binding with Pbx1 (Zákány and Duboule, 1999). Thus, the distal fore and hind limbs, which are specified by Hox proteins lacking Pbx dimerization motifs (paralogs 11-13), show no morphological abnormalities in Pbx1−/− embryos. This correlation, together with the various Pbx1−/− non-skeletal phenotypes overlapping Hox mutant phenotypes (Table 2), indicates that at least one role for Pbx1 in vivo is likely to be that of a selective Hox cofactor, whose actions are delimited by its capacity to dimerize with some, but not all, members of the Hox family. Consistent with this role, Pbx1−/− embryos show unperturbed spatial and temporal expression profiles of a representative subset of Hox genes that normally contribute to the specification of structures affected by the absence of Pbx1.

In spite of the associations noted above, our observations that the skeletal malformations associated with loss of Pbx1 do not precisely recapitulate individual Hox gene deficiencies raises the possibility of Hox-independent roles for Pbx1 in patterning. Indeed Pbx1 participates in diverse transcription complexes, only some of which are known to contain Hox components. However, overlapping expression of highly similar members of the respective Hox and Pbx protein families creates difficulty in unambiguously assigning specific roles. Single or compound Hox null phenotypes represent not only loss of individual Hox function, but also the summation of relative overlapping contributions from other Hox genes that are co-expressed in the affected body segments (Krumlauf, 1994). Comparable Hox compensation, however, is unlikely to modulate the Pbx1 phenotype if Pbx1 globally compromises the actions of multiple Hox proteins that contribute to specification of individual body segments. It is also possible that loss of Pbx1 mimics a hypomorphic rather than null Hox phenotype, since not all Hox functions may be Pbx1 dependent. Finally, other members of the redundant Pbx family (Monica et al., 1991) may overlap with Pbx1 in their expression profiles and thus compensate for the loss of Pbx1 and ameliorate the nullizygous phenotypes.

Loss of Pbx1 is accompanied by a homeotic-like transformation in the second branchial arch (BA2) where, for example, the lesser horns of the hyoid are transformed into structures reminiscent of BA1-derived Meckel’s cartilages. Similarly, in the zebrafish Lazarus mutant, BA2 cartilages, such as the hyosymphetic, are more similar to BA1 cartilages, such as the palatoquadrate (Popperl et al., 2000). We considered four possible mechanisms that may have contributed to the anterior-like transformation of BA2 to BA1-like morphology. These include loss of function of Hoxa2 protein in BA2, loss of expression of genes normally expressed in BA2 but not in BA1 (e.g. Hoxa2), gain of expression in BA2 of genes normally restricted to BA1 (e.g. Lhx6), and misexpression of genes normally proximodistally restricted within BA2 (dHAND, Gsc). Our data support the first scenario, since we observed no clear alterations in Hoxa2 expression at E10.5. Like the Pbx1−/− mouse embryos, Hoxa2 null mutants also display an anteriorization of the second arch skeleton (Fig. 7C), although there are differences in the nature of the morphologic transformation (Rijli et al., 1993). One explanation for the morphological differences between these mutants is that both genes have independent, as well as dependent functions in modifying the mandibular arch program to generate the hyoid arch program. Anterior transformation of second branchial arch-derived structures in Pbx1−/− embryos provides further support to the idea that the splanchnocranium of the first and second arches share common developmental programs (Rijli et al., 1993).

Pbx1 is obligatory for patterning the proximal-distal limb axis in vertebrates

Our results demonstrate that Pbx1 is essential for patterning the proximal limbs in vertebrates, as its invertebrate homolog exd is required for patterning the proximal limbs of Drosophila (Gonzales-Crespo et al., 1998). In the fly limb, exd RNA and protein are broadly expressed, whereas nuclear localization of EXD protein is restricted to proximal regions (González-Crespo et al., 1998). In contrast, patterning of the distal fly leg, where EXD is cytoplasmic, is dependent on signaling by wingless and decapentaplegic, which are in turn activated by hedgehog (González-Crespo et al., 1998). In the mouse, the subcellular localization of Pbx1 in the developing limb parallels that of EXD in the Drosophila leg (González-Crespo et al., 1998; this study). This has led to the suggestion that the regulation of nuclear availability of Pbx/EXD cofactors controls the execution of Hox programs in limb development.

Recent studies provide a molecular framework for the
control of proximal-distal limb specification and outgrowth based on antagonistic interactions conserved from insects to vertebrates. Ectopic expression of Meis genes in the distal limb of chicks induced proximalization of distal structures (Capdevila et al., 1999; Mercader et al., 1999). This suggested that normal restriction of Meis, and its invertebrate homolog homothorax (HTH), to the proximal regions of the vertebrate and insect limb, where Pbx1 is co-localized in the nucleus, is essential to specify cell fates and differentiation patterns along the proximal-distal axis. However, the complexity of Hox functions, their cross-regulation, and interactions with Meis/Pbx, make it difficult to conclusively interpret these ectopic expression data (reviewed in Vogt and Duboule, 1999). Conversely, the zebrafish Lazarus mutant dies very early, before vertebrae, posterior trunk and posterior fins develop (Popperl et al., 2000), making it impossible to assess the role of Pbx in proximal-distal limb specification in zebrafish. Our study unequivocally demonstrates that proximal-distal limb patterning in vertebrates, which is proposed to be controlled by Meis (Capdevila et al., 1999; Mercader et al., 1999), is dependent on the obligatory presence of Pbx1 in the proximal limb domain.

Requirement for Pbx1 in programming chondrocyte proliferation, terminal differentiation, and bone formation

Pbx1 mutants display defects in the development of specific endochondral skeletal elements. Endochondral bone development is a complex and tightly regulated process that requires programmed proliferation and maturation of chondrocytes followed by terminal differentiation, hypertrophy and replacement of cartilage by bone (reviewed by Karsenty, 1999; Olsen et al., 2000). Although Pbx1−/− cells appear to be capable of executing a complete program of endochondral ossification, as evidenced by the deposition of bone matrix in affected skeletal domains, the regulation of this process in affected elements is perturbed. Prior to E13.5, no clear histological differences were observed in the abilities of Pbx1−/− mesenchymal cells to condense and differentiate into proliferating chondrocytes. Similarly, at E13.5 histological analysis of both wild-type and Pbx1−/− embryos showed comparable collections of proliferating chondrocytes modeling the outlines of nascent skeletal elements. However, measurements of BrdU incorporation in vivo showed marked reduction in proliferation of Pbx1−/− chondrocytes at E13.5 and E14.5. By E15.5, the ribs of Pbx1−/− mutants completely lacked proliferating (PCNA+) chondrocytes. Furthermore, histological and immunohistochemical studies show that chondrocytes of altered non-splanchnocranial elements had precociously matured to the terminal stage of chondrogenesis. Thus, lack of Pbx1 impacted the normal progression of chondrocyte terminal differentiation, resulting in disproportionate numbers of hypertrophic chondrocytes and precocious cartilage mineralization and bone formation. Whole embryo cartilage preparations also support this conclusion (data not shown). Performing whole embryo cartilage/bone staining at earlier days (E12.5-E14.5) could provide further support. Analogous perturbations in chondrocyte maturation may also have contributed to hyoid bone transformation, but in the form of enhanced, as opposed to attenuated, proliferation. The findings in Pbx1−/− mutant cartilage parallel our observations that the hematopoietic defect in Pbx1−/− embryos results in part from deficiencies in the proliferative expansion of select hematopoietic progenitor cell populations (DiMartino et al., 2001). Thus, Pbx1 may serve similar roles to orchestrate the growth and proliferation of progenitors at specific developmental stages within multiple cellular lineages.

Pbx1 does not have an upstream role in controlling the PTHrP-Ihh pathway that regulates chondrogenesis

The defective maturation and terminal differentiation of chondrocytes in Pbx1 mutants could be the consequence of perturbations of the timing and/or extent of chondrocyte proliferation arising from altered expression of regulators of chondrogenesis. Many of the key regulatory molecules have been identified and insights into their roles gained from analyses of the impact of loss- or gain-of-function mutations on endochondral ossification in humans and mice (reviewed by Olsen et al., 2000). Such studies have shown that PTHrP and PTHrPR are important regulators of chondrocyte maturation acting to delay hypertrophy of chondrocytes. Loss of function in mice results in premature maturation of chondrocytes leading to excessive bone formation at birth (Karaplis et al., 1994; Vortkamp et al., 1996; Lanske et al., 1996). Although these phenotypes are similar histologically to those observed in the Pbx1−/− mutants, the endochondral bone development defect is widespread in PTHrP null mice (Karaplis et al., 1994), while it is restricted to specific skeletal segments in the absence of Pbx1.

Regulation of chondrogenesis by the PTHrP/PTHrPR pathway also involves other key regulators such as Sox9 and Ihh. The high-mobility-group domain transcription factor, Sox9, expressed throughout all cartilaginous condensations, is required for chondrogenesis (Bi et al., 1999). Recent studies suggest that Sox9 is a target of PTHrP signaling and that it may mediate some effects of PTHrP in the growth plate, inhibiting the maturation of pre-hypertrophic chondrocytes to hypertrophic chondrocytes (Huang et al., 2000). Ihh is a key signaling molecule, which stimulates chondrocyte proliferation and prevents chondrocyte hypertrophy through PTHrP-dependent and -independent pathways (St-Jacques et al., 1999; Vortkamp et al., 1996; Lanske et al., 1996). Lack of Ihh results in greatly reduced chondrocyte proliferation, delayed maturation, and poor bone formation. These changes in Ihh mutants are widespread in the skeleton, in contrast to the restricted distribution in Pbx1 mutants (St-Jacques et al., 1999). Thus, Ihh and Pbx1 mutants are very distinct from each other. Fgfr3-deficient mice also show defects in endochondral bone development. Unlike Pbx1 mutants, they exhibit extended growth plates and striking overgrowth of the long bones (Colvin et al., 1996; Deng et al., 1996), thus revealing a function for Fgfr3 as a general negative regulator of chondrocyte proliferation. MMP-9 is also a key regulator of chondrogenesis but unlike Pbx1, the MMP-9 defect is restricted to the growth plate, and hypertrophic chondrocytes develop normally but ossification is delayed (Vu et al., 1998). Thus, despite some similarities, most features of the Pbx1 mutant phenotype are distinct from those arising from loss of function of these known regulators of skeletogenesis. Furthermore, no major differences in the spatial or temporal expression of these chondrogenic regulators (Ihh, PTHrP,
PTHrPR, Sox9 and Fgfr3) were detected in Pbx1−/− rib cartilages.

Taken together, these results strongly suggest a model in which Pbx1 is required to coordinate diverse facets of embryonic skeletogenesis via mechanisms that are likely distinct from known molecular pathways involving PTHrP/PTHrPR/IIh/Fgfr3. The lack of major differences in expression of these regulators in Pbx1 mutants indicates that Pbx1 is unlikely to be an upstream regulator of these genes at early stages of skeletal morphogenesis. Thus, a scenario could be envisaged where either Pbx1 functions downstream of these regulators of chondrogenesis, or alternatively Pbx1 may regulate chondrogenesis via collaborative interactions with other co-expressed factors that function in concert or in parallel, such as Sox9 or Hox proteins. Indeed, specificity of action by Sox9 is achieved via interactions with specific partner factors (reviewed by Kamachi et al., 2000). Moreover, combinatorial interactions among Pbx, Prep or Hox proteins have been shown to be involved in regulating tissue-specific production of collagen V (Penkov et al., 2000).

Given that we observed perturbations only in skeletal domains specified by Hox proteins bearing Pbx dimterization motifs, our results provide strong evidence that the requirement for Pbx1 in programming chondrocyte proliferation and maturation is Hox dependent. Indeed, a potential role of Hox proteins in the regulation of progenitor cell proliferation has been previously suggested based on studies of Hox null mice (Condie and Capecchi, 1993; Condie and Capecchi, 1994; Duboule, 1995). Furthermore, studies using retroirral vectors to misexpress Hox genes in chick limb bud (Goff and Tabin, 1997) have shown that Hoxd11 and Hoxd13 pattern the limb skeleton by regulating the rates of cell division in the proliferative zone of growing cartilage. Thus, a model in which Hox genes act as growth promoters has also been proposed (Goff and Tabin, 1997). Lastly, over-expression of a Hoxc8 transgene has been shown to cause cartilage defects characterized by an accumulation of proliferating chondrocytes and reduced maturation (Yueh et al., 1998).

In summary, our studies reveal a previously unknown function for Pbx1 as a component of the cellular machinery that determines the temporal coordination of progenitor cell proliferation and differentiation in vertebrates. Elucidation of the molecular basis for chondrogenic defects using intact animal models, such as the Pbx1-deficient mouse used here, will contribute to a better understanding of the different pathways that regulate skeletal development in vertebrates.

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