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Abnormalities of caudal pharyngeal pouch development in *Pbx1* knockout mice mimic loss of *Hox3* paralogs

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Abstract

Pbx1 is a TALE-class homeodomain protein that functions in part as a cofactor for Hox class homeodomain proteins. Previous analysis of the in vivo functions of *Pbx1* by targeted mutagenesis in mice has revealed roles for this gene in skeletal patterning and development and in the organogenesis of multiple systems. Both RNA expression and protein localization studies have suggested a possible role for Pbx1 in pharyngeal region development. As several *Hox* mutants have distinct phenotypes in this region, we investigated the potential requirement for Pbx1 in the development of the pharyngeal arches and pouches and their organ derivatives. *Pbx1* homozygous mutants exhibited delayed or absent formation of the caudal pharyngeal pouches, and disorganized patterning of the third pharyngeal pouch. Formation of the third pouch-derived thymus/parathyroid primordia was also affected, with absent or hypoplastic primordia, delayed expression of organ-specific differentiation markers, and reduced proliferation of thymic epithelium. The fourth pouch and the fourth pouch-derived ultimobranchial bodies were usually absent. These phenotypes are similar to those previously reported in $Hoxa3^{-/-}$ single mutants and $Hoxa1^{-/-}$;$Hoxb1^{-/-}$ or $Hoxa3^{+/-}$;$Hoxb3^{-/-}$;$Hoxd3^{-/-}$ compound mutants, suggesting that Pbx1 acts together with multiple Hox proteins in the development of the caudal pharyngeal region. However, some aspects of the *Pbx1* mutant phenotype included specific defects that were less severe than those found in known *Hox* mutant mice, suggesting that some functions of Hox proteins in this region are Pbx1-independent.

Keywords: Pbx; Hox; Pharyngeal arches; Pharyngeal pouches; Thymus; Parathyroids; Organogenesis

Introduction

In mammalian embryogenesis, the biological functions of the homeobox gene *Pbx1*, initially identified at the site of a chromosomal translocation in acute pediatric leukemias (Kamps et al., 1990; Nourse et al., 1990), remain poorly understood in comparison to its well-characterized role as a DNA-binding partner with Hox proteins (Mann and Affolter, 1998). Pbx1 is a three amino acid loop extension (TALE) class homeodomain protein (Burglin, 1997) that can also dimerize with other TALE homeodomain proteins, such as Meis/Prep (Berthelsen et al., 1999). As a TALE heterodimer, Pbx1 displays an ability to simultaneously bind DNA with a subset of *Hox* proteins and can form trimeric complexes on appropriate DNA sites (Ferretti et al., 2000; Jacobs et al., 1999). Numerous studies have demonstrated that Pbx1–Hox interactions result in enhanced DNA binding affinities and specificities in vitro (Chan et al., 1994, 1997; Chang et al., 1995, 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.

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Genetic analyses in both mice and *Drosophila* provide in vivo support that Pbx and exd proteins (Peifer and Wieschaus, 1990; Rauskolb 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 and developmental programs in multiple tissues.

Our recent studies demonstrate a critical role for Pbx1 as a developmental regulator, whose absence results in embryonic lethality at around gestational days 15–16 (E15–16) with multiple tissue and organ system abnormalities (Selleri et al., 2001). Specifically, we demonstrated that Pbx1 is required for patterning and skeletal development (Selleri et al., 2001), maintenance of definitive hematopoiesis in the fetal liver (DiMartino et al., 2001), pancreatic development and function (Kim et al., 2002), and urogenital and adrenal organ development (Schnabel et al., 2003a,b).

Because of the known interactions of Pbx and Hox proteins, clues to additional roles of Pbx1 in development may come from *Hox* gene mutants (Krumlauf, 1994). *Pbx1* is expressed in the pharyngeal region during embryonic development (Schnabel et al., 2001), and several single and multiple *Hox* mutants have phenotypes in this region (Chisaka and Capecchi, 1991; Manley and Capecchi, 1995, 1998; Rossel and Capecchi, 1999). The vertebrate pharyngeal region is the source of the endoderm-derived glandular organs, the thyroid, ultimobranchial bodies, thymus, and parathyroids, which play a variety of essential physiological roles. The pharyngeal organs all originate from out-pocketings of the pharyngeal endoderm, either in the form of pharyngeal pouches or the thyroid diverticulum, and require interactions between the endoderm and surrounding mesenchyme for their development (Grapin-Botton and Melton, 2000). The pharyngeal pouches are transient, bilateral out-pocketings of the pharyngeal endoderm that form in an anterior to posterior order beginning at about E8.5. Each of the pouches contacts the surface ectoderm about 1 day after formation, via ectodermal invaginations called pharyngeal clefts. The pharyngeal pouches are directly surrounded by pharyngeal arch mesenchyme, which is primarily composed of neural crest cells (NCCs), although there is also a mesodermal component in the center of each arch (LeLievre and LeDouarin, 1975). Experimental and genetic evidence indicates that both endoderm and NCC mesenchyme are directly involved in the development of all of these pharyngeal organs (Grapin-Botton and Melton, 2000; Manley and Blackburn, 2003).

The best-characterized *Hox* mutant with pharyngeal region defects is *Hoxa3*. *Hoxa3* mutants have normal initial pharyngeal pouch formation, but have severe defects in pharyngeal organ development, including athymia, aparathyroidism, and thyroid hypoplasia, in addition to a broad spectrum of neural, skeletal, and cardiovascular defects (Chisaka and Capecchi, 1991; Kameda et al., 2002; Manley and Capecchi, 1995). Mice carrying mutations in all of the *Hox3* paralogs ($Hoxa3^{+/-}$;$Hoxb3^{-/-}$;$Hoxd3^{-/-}$) have ectopic thymus and parathyroids (Manley and Capecchi, 1998). Furthermore, mice mutant for both the *Hoxa1* and *Hoxb1* genes have severe defects in both posterior pouch formation and pharyngeal organ development (Rossel and Capecchi, 1999). Taken together, these results indicate that *Hox* genes play roles in both formation and patterning of the posterior pharyngeal pouches and their organ derivatives. It is currently unclear, however, which potential cofactors may contribute to or modify the Hox program in these sites.

To address Pbx1 function in vertebrate pharyngeal pouch formation, patterning, and organ development, mice homozygous for a null allele of *Pbx1* (Selleri et al., 2001) were examined for pharyngeal region phenotypes. In the current study, we show that $Pbx1^{-/-}$ mutant embryos exhibited phenotypes that are similar to or more severe than abnormalities in pharyngeal development caused by single or multiple *Hox* gene mutations, and suggest that Pbx1 acts collaboratively with several different Hox proteins in the development of the pharyngeal region. Furthermore, some aspects of the *Pbx1* mutant phenotype are not as severe as reported *Hox* mutant pharyngeal phenotypes, suggesting either that interactions with Pbx proteins are not required for all *Hox*-dependent aspects of pharyngeal development, or that other Pbx proteins are providing redundant function in this region.

Materials and methods

Generation and genotyping of $Pbx1^{-/-}$ mice

Targeted disruption of *Pbx1* and genotyping of embryos by PCR and Southern hybridization were performed as described (Selleri et al., 2001). Phenotypes were analyzed in mice derived from either of two independently targeted lines that were generated on a mixed 129/Sv-C57BL/6J genetic background. *Pbx1* heterozygous mice were born at the expected Mendelian ratios and did not display any anatomical abnormalities, except reduced weight (Kim et al., 2002; Selleri et al., 2001).

Histology and immunohistochemistry

For histological analysis, embryos were fixed in formalin and embedded in paraffin for sectioning using standard procedures. Five-micron sections were stained with hematoxylin and eosin, mounted in DPX and photographed using a Magnafire digital camera (Optronix). Immunohistochemistry was performed on dewaxed paraffin sections following microwave antigen retrieval (Schnabel et al., 2001). The primary antibody used to detect Pbx1 protein was a mouse Pbx1b-specific monoclonal antibody (αPbx1b; (Jacobs et

al., 1999). Specificity of the antibody was previously demonstrated by lack of signal on *Pbx1* homozygous null embryos (Schnabel et al., 2003a; Selleri et al., 2001).

Analysis of BrdU incorporation

Pregnant mice were injected intravenously with BrdU at a dose of 50 μg/g of body weight 1 h before sacrifice. Embryos were fixed and embedded in paraffin as above to obtain transverse sections. BrdU was detected by immunohistochemistry as described (Nowakowski et al., 1989; Selleri et al., 2001) and sections were counterstained lightly with hematoxylin and eosin. Monoclonal anti-BrdU was purchased from Roche. All BrdU-positive (dark brown) and negative (blue) nuclei were counted over various sections. At least five different sections containing thymic primordia were analyzed for each wild-type and *Pbx1*$^{-/-}$ littermate embryo (2 wild-type and 4 *Pbx1*$^{-/-}$ at E12.5; 2 wild-type and 2 *Pbx1*$^{-/-}$ at E11.5 and 1 wild-type and 1 *Pbx1*$^{-/-}$ at E13.5). Sections were digitized using an Eclipse E600 microscope (Nikon). For each section, outlines of the thymic lobes were traced manually, corresponding areas were computed and positive and negative cells counted digitally (Stereo Investigator Software Package, Version 5.05.4, Microbrightfield). Approximately 200 total cells were counted per each section, and right and left thymic lobes were analyzed independently.

Whole mount in situ hybridization

Whole mount in situ hybridization was performed as described (Carpenter et al., 1993; Manley and Capecchi, 1995), using *Pbx1*$^{-/-}$ and littermate control embryos. The *Hoxa3, Pax1* (Manley and Capecchi, 1995), *Gcm2, Foxn1* (Gordon et al., 2001), *AP2* (Rossel and Capecchi, 1999), and *Tbx1* (Jerome and Papaioannou, 2001) in situ hybridization probes have been previously described.

Results

Pbx1$^{-/-}$ *mutants have defects in pharyngeal organ formation*

As an initial analysis of pharyngeal region development in *Pbx1*$^{-/-}$ embryos, we performed a histological assessment of pharyngeal pouch derivatives in E11.5–E15.5 *Pbx1*$^{-/-}$ and littermate control embryos. In wild-type embryos, the second pouch degenerates by E12 without forming any further structures. Each third pouch gives rise to a single organ primordium that forms at about E11 and then separates into one thymic lobe and one parathyroid gland by E13.5 (Manley and Blackburn, 2003). The fourth pouch derivatives are the ultimobranchial bodies, which form at E11.5 and fuse with the thyroid diverticulum beginning at E13.5 to contribute calcitonin-producing C cells to the thyroid gland. A summary of the third and fourth pouch-derived organ phenotypes in the *Pbx1* mutants is shown in Table 1.

At E11.5 in control embryos, the second pouch was still present but beginning to degenerate (data not shown), while the third and fourth pouch-derived organ primordia had begun to form, but were still attached to the pharynx (Figs. 1A, D). In all three of the *Pbx1*$^{-/-}$ mutants examined histologically at E11.5, third pouch derived organ primordia were present on both sides, but showed variable hypoplasia compared to controls (Figs. 1B, C). In one embryo, one of the third pouch primordia was also fused to the second pouch (data not shown). Other than this one case, the second pouches appeared normal in all mutant embryos examined (data not shown). The most severe defects were seen in the fourth pouch, where all three embryos analyzed had missing or small fourth pouches, with no evidence of ultimobranchial body formation (Fig. 1E).

In control embryos at E12.5, the third and fourth pouch-derived organ derivatives had separated from the pharynx

Table 1
Summary of *Pbx1*$^{-/-}$ pharyngeal pouch-derived organ phenotypes

Stage	Thymus phenotype	Parathyroid phenotype	Ultimobranchial body phenotype
E11.5	3/3 bilateral hypoplastic[a,b]	3/3 bilateral hypoplastic[a,c]	3/3 absent
E12.5	1/6 bilateral hypoplastic	2/6 bilateral	2/6 unilateral hypoplastic
	2/6 bilateral hypoplastic, ectopic	2/6 unilateral	3/6 absent
	3/6 unilateral, ectopic	2/6 not scorable[d]	1/6 not scorable[d]
E13.5	2/5 bilateral hypoplastic, ectopic	1/5 bilateral	5/5 absent
	1/5 unilateral	4/5 absent	
	2/5 absent		
E15.5	2/3 bilateral hypoplastic, ectopic	2/3 bilateral, ectopic	N.D.[e]
	1/3 absent	1/3 absent	

[a] E11.5 embryos were scored for the third pouch common thymus/parathyroid primordia. Size of the primordia when present varied, but was always clearly smaller than littermate controls.
[b] Of four other *Pbx1*$^{-/-}$ embryos assayed for *Foxn1* expression at this stage, one had no detectable expression, two had unilateral expression, one had expression on one side with the other side lost in processing. *Foxn1*-positive lobes had only very small expression domains (see Fig. 6).
[c] Of three other *Pbx1*$^{-/-}$ embryos assayed for *Gcm2* expression at this stage, two had bilateral expression and one had unilateral expression (see Fig. 6).
[d] Embryos listed as not scorable had damaged or missing sections that precluded definitive evaluation of the phenotype.
[e] Not Detected—since ultimobranchial bodies are usually fused to the thyroid at this stage, the only phenotype detectable at this stage is persistent or unfused organs, which were never seen in these mutants.

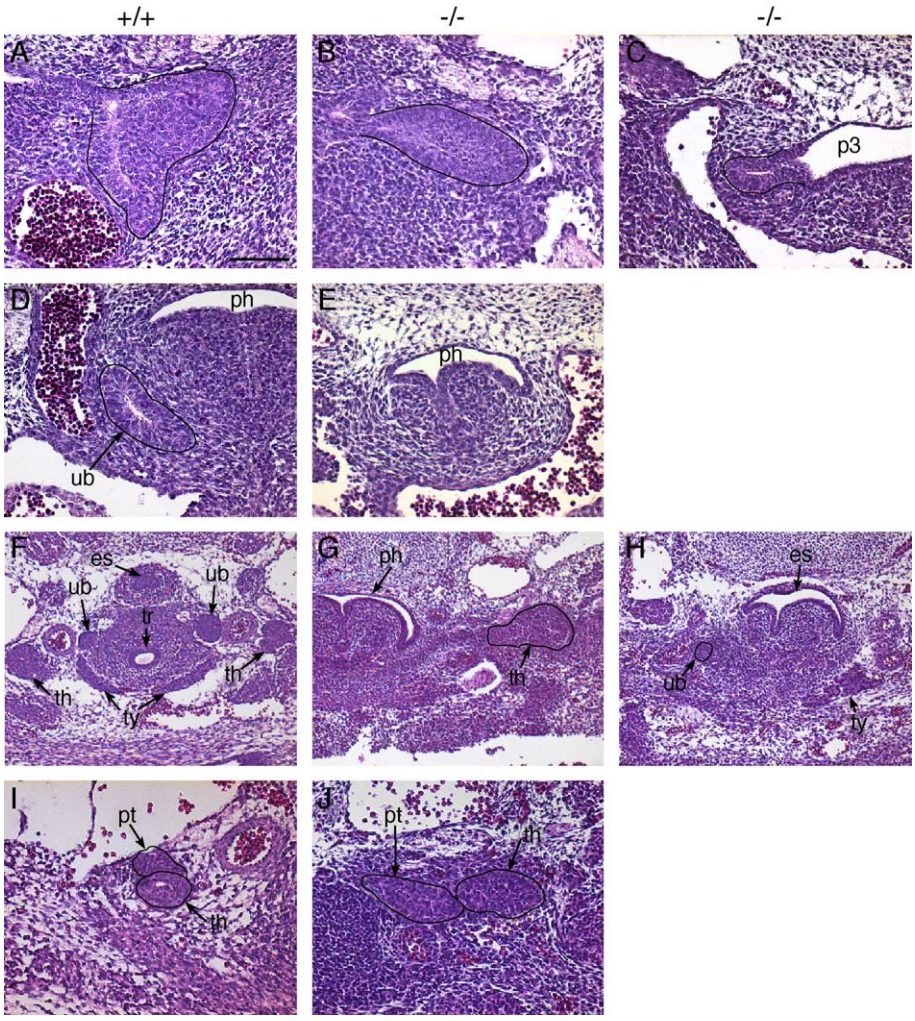

Fig. 1. Pharyngeal organ phenotypes in *Pbx1*$^{-/-}$ embryos at E11.5–E12.5. Transverse H&E-stained sections of E11.5 (A–E) and E12.5 (F–J) wild-type (A, D, F, I) or *Pbx1*$^{-/-}$ (B, C, E, G, H, J) embryos. Organ primordia in A–D and G–J are outlined in black. (A) The common thymus/parathyroid primordium in a wild-type embryo, still attached to the pharynx at this stage. (B) An example of a mild *Pbx1*$^{-/-}$ phenotype, with a well-formed but hypoplastic primordium. The other side of this embryo had no primordium formation. (C) Example of a severely hypoplastic third pouch (p3) primordium from a second littermate mutant embryo. (D) Ultimobranchial body (ub) in a wild-type embryo. The pharynx (ph) at this stage has not yet developed a separate trachea and esophagus. (E) Section of a mutant embryo in the general position where a ub should have formed showed no primordium formation. (F) Wild-type embryo showing thymic lobes (th), ubs, and ventral thyroid (ty). Note that the esophagus (es) and trachea (tr) are well developed and are separated at this level. (G) Ectopic but normally sized thymic lobe in a *Pbx1*$^{-/-}$ littermate. The thymus is located above the esophageal–tracheal split. No thymus or parathyroid was present on the other side of this embryo. (H) A second *Pbx1*$^{-/-}$ littermate with a thyroid only on one side and a possible ub on the other. Note the abnormal morphology of the esophagus. (I) A more anterior section from the same embryo as in (F), showing the parathyroid and anterior tip of the thymus. (J) Ectopic parathyroid and thymus from the same embryo as in (H). The apparent difference in size is due to a slight difference in the plane of section. This embryo had only one thymus and parathyroid. Scale bars: (A–E, I, J) 30 μm; (F–H) 60 μm.

and begun migrating ventrally and caudally. The thymic lobes in particular were significantly larger (Fig. 1F), and contained developing T cells (thymocytes) as identified by histological analysis (not shown). Ultimobranchial bodies were associated with the anterior and dorsal aspects of the thyroid lobes, but had not yet begun to fuse with the thyroid (Fig. 1F). Of the six *Pbx1*$^{-/-}$ mutant embryos examined by histology at this stage, three had only a single ectopic thymus/parathyroid rudiment (Fig. 1G). In the other three mutants, two thymus/parathyroid rudiments were present, all of which were severely hypoplastic, and in two embryos were both ectopic, consistent with defective organ migration. Although the parathyroids at this stage are normally closely associated with the thymic lobes, they were readily identified in all control embryos assayed (Fig. 1I). Parathyroid development was identified in all four scorable mutants at this stage (Fig. 1J). Consistent with the abnormal fourth pouch development at E11.5, only two of five E12.5 embryos scored for this phenotype had a single, very small fourth pouch derivative (Fig. 1H).

Phenotypes observed at later stages were similar to those seen at E12.5. At E13.5 in control embryos, the ultimobranchial bodies had begun to fuse with the thyroid lobes, and the parathryoids were located lateral to the thyroid

gland (Fig. 2A). The thymic lobes were much larger than at E12.5, had descended to the posterior aspect of the thyroid, and were located more medially (Fig. 2B). In contrast, two out of five *Pbx1* mutants had no pharyngeal pouch-derived organs (Fig. 2D), and one had only a single, relatively normal sized but ectopically located thymic lobe (Fig. 2C). The remaining two did have bilateral thymic rudiments, although they were extremely hypoplastic (data not shown). No evidence of ultimobranchial bodies was found at this stage. Furthermore, the thyroid glands in at least three mutants were very small and poorly formed (Fig. 2C). It is not clear whether this thyroid phenotype was a primary defect, or was secondary to the absence of ultimobranchial bodies, which normally begin to fuse with the thyroid at this stage. At the latest stage observed, E15.5, one of three mutant embryos showed no pouch-derived organs, while the remaining two had bilateral, hypoplastic, and ectopic thymic lobes and contained bilateral and ectopic parathyroids (Table 1 and data not shown).

Proliferation is reduced in the early thymic/parathyroid rudiment

Both *Pbx1* (DiMartino et al., 2001; Kim et al., 2002; Selleri et al., 2001) and *Hox* genes (Condie and Capecchi, 1993, 1994; Duboule, 1995; Goff and Tabin, 1997; Su et al., 2001; Yueh et al., 1998) have been implicated in regulating proliferation. To investigate whether the smaller thymic rudiments were the result of reduced proliferation, we used BrdU incorporation to measure proliferation in two mutant and two littermate control embryos during early organogenesis at E11.5–12.5. Both control embryos taken at E11.5 had large bilateral third pouch-derived primordia, with 25–30% BrdU-positive cells (Figs. 3A, D). As was expected from the histological analysis, in both of the mutants examined only one third pouch-derived lobe was present. In one case, an epithelial structure that was likely a remnant of the third pouch was present on the other side, but contained no BrdU-positive cells (Fig. 3C), and in the other mutant embryo, no signs of organ formation were present on one side. In the one lobe that was present in each mutant embryo, proliferation was consistently decreased in the thymic rudiment by approximately 50% (Figs. 3B, D). As this stage is prior to significant lymphocyte progenitor infiltration of the rudiment, this decrease in proliferation is most likely to be a primary defect in the thymic epithelium or mesenchyme.

At E12.5, both thymic lobes in the control embryo had about 40% BrdU-positive cells (Fig. 3D). This increase in proliferating cells relative to E11.5 was likely due in part to the presence of proliferating hematopoietic-derived lymphocyte progenitor cells (LPCs), which have begun to infiltrate the thymus at this stage. LPCs were also present in the mutant thymic lobes, which could have masked any change in epithelial cell proliferation. However, two of the three thymic lobes counted from $Pbx1^{-/-}$ embryos had

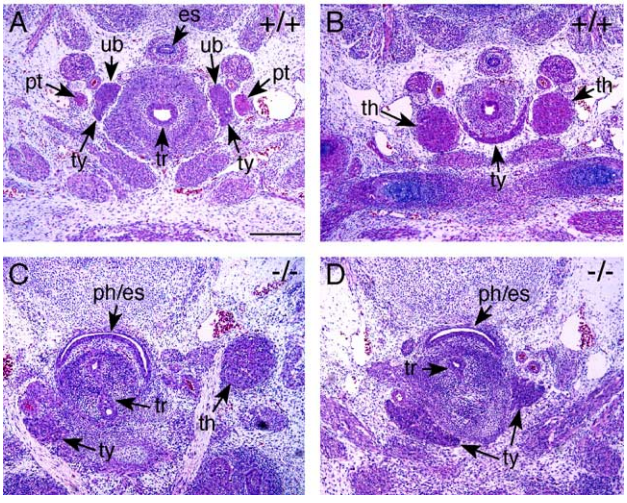

Fig. 2. Pharyngeal organ phenotypes in $Pbx1^{-/-}$ mice at E13.5. Transverse H & E stained sections of one wild-type (A, B) and two different littermate $Pbx1^{-/-}$ (C, D) embryos. (A) Section showing the parathyroids (pt), and the ultimobranchial bodies (ub) fusing to the dorsal anterior thyroid (ty). Note the well-developed esophagus (es) and trachea (tr). (B) A more posterior section of the same embryos in (A), showing the thymic lobes (th) and the ventral thyroid. (C) $Pbx1^{-/-}$ littermate with a single, ectopic thymic lobe and thyroid on the opposite side. Note the abnormal pharyngeal morphology and failure of esophageal differentiation (ph/es). This section is in a slightly coronal plane, resulting in an abnormal appearance of the trachea. (D) A second $Pbx1^{-/-}$ littermate with a bilateral thyroid but no parathyroids, thymic lobes, or ubs. Scale bars: 50 μm.

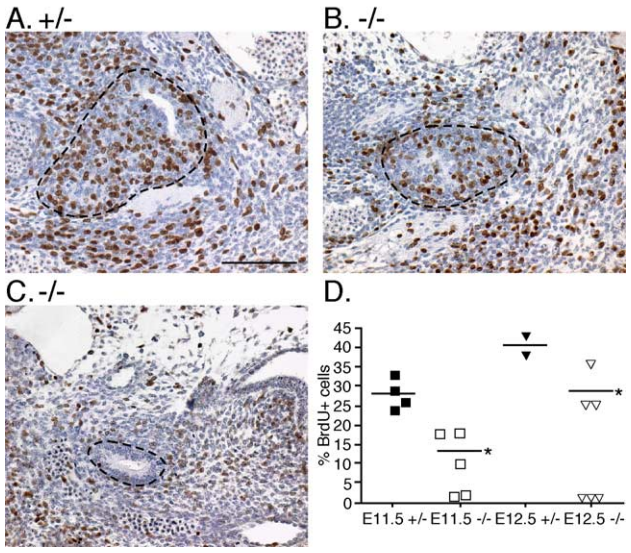

Fig. 3. Reduced proliferation in $Pbx1^{-/-}$ embryos at E11.5. Proliferation was assayed by BrdU incorporation (brown cells). Cell counts were performed on transverse paraffin sections counterstained with hematoxylin. (A) $Pbx1^{+/-}$ thymic lobe section with 33% BrdU-positive cells. (B) Section of a thymic lobe in a $Pbx1^{-/-}$ littermate with 19% BrdU incorporation. (C) Very small thymic lobe from another $Pbx1^{-/-}$ embryo with few or no BrdU-positive cells. (D) Scatterplots of average BrdU-positive cell counts per thymic lobe, with means indicated. Average values in the two mutant columns (*) were calculated excluding the thymic lobes that were absent or had no BrdU-positive cells. Scale bar: 100 μm.

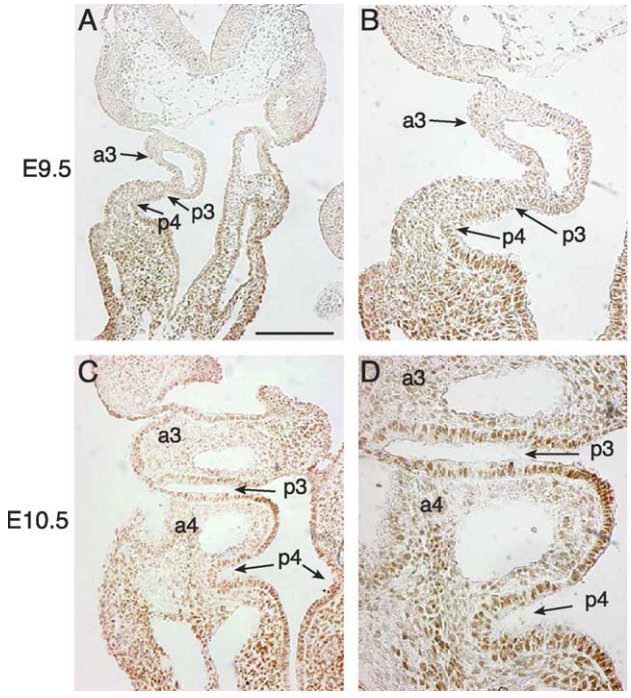

Fig. 4. Pbx1 nuclear localization in the third and fourth pharyngeal pouch region. Coronal sections stained by immunohistochemistry for Pbx1 in wild-type embryos at E9.5 (A, B) and E10.5 (C, D). B and D show higher magnification pictures of A and C. Nuclear localized Pbx1 is most prominent in the mesenchyme, endoderm, and ectoderm of the fourth arch at E9.5. At E10.5, nuclear Pbx1 is most clearly seen in the pouch endoderm and surface ectoderm, as well as in subpopulations of mesenchyme. a3, third arch; a4, fourth arch; p3, third pouch; p4, fourth pouch. Scale bars: (A, C) 85 μm; (B, D) 170 μm.

proliferation levels that were reduced to 60% of wild type levels (Fig. 3D), indicating that either the epithelial cells or LPCs or both had reduced proliferation at this stage.

Nuclear localized Pbx1 in the pharyngeal endoderm and mesenchyme

We previously reported that nuclear-localized Pbx1 protein is present within the anterior pharyngeal region at E9.5–10.5 (Schnabel et al., 2001; Selleri et al., 2001), and in the mesenchyme of the second pharyngeal arch at E11.5, while the mesenchyme of the mandibular and maxillary branches of the first arch show much lower levels of *Pbx1* expression (Selleri et al., 2001). However, these analyses were focused on the first and second pharyngeal arches, rather than the more caudal arches and pouches, which are the origins of the pharyngeal organs that were affected in the *Pbx1* mutants. Furthermore, the phenotype found in the current study suggested that development of the caudal pouches was defective in *Pbx1*$^{-/-}$ embryos. Therefore, we re-analyzed the localization of Pbx1 protein in wild type E9.5 and E10.5 embryos, focusing on the caudal pharyngeal region.

Nuclear Pbx1 protein was present in the surface ectoderm, pharyngeal pouch endoderm, and in subsets of arch mesenchyme at both E9.5 and E10.5 (Fig. 4). At E9.5, nuclear localized protein levels were higher in the region that will form the fourth arch and pouch in all cell types, with graded expression anterior to this region (Figs. 4A, B). By E10.5, the highest levels of nuclear localized Pbx1 protein were seen in the surface ectoderm and pouch endoderm throughout the pharyngeal region (Figs. 4C, D). At this stage, mesenchymal localization was more prominent in subsets of mesenchyme in arches three and four. Nuclear-localized Pbx1 was least prominent in the mesenchyme immediately surrounding the third pouch in the region where the thymus/parathyroid primordia form (Fig. 3). This localization combined with the phenotypes observed suggests that Pbx1 may be acting in multiple cell types in the region. In particular, the more extensive and earlier presence of nuclear-localized Pbx1 protein in the region of the developing fourth pouch correlates with the loss of fourth pouch formation. In contrast, pharyngeal endoderm expression may be more important for the third pouch phenotypes.

Marker gene analysis indicates defects in pouch formation and patterning

Our histological analysis indicated that the *Pbx1*$^{-/-}$ embryos had defects in the patterning and/or formation of the pharyngeal pouches. We used marker gene analysis to further investigate the possible changes in genetic regulatory

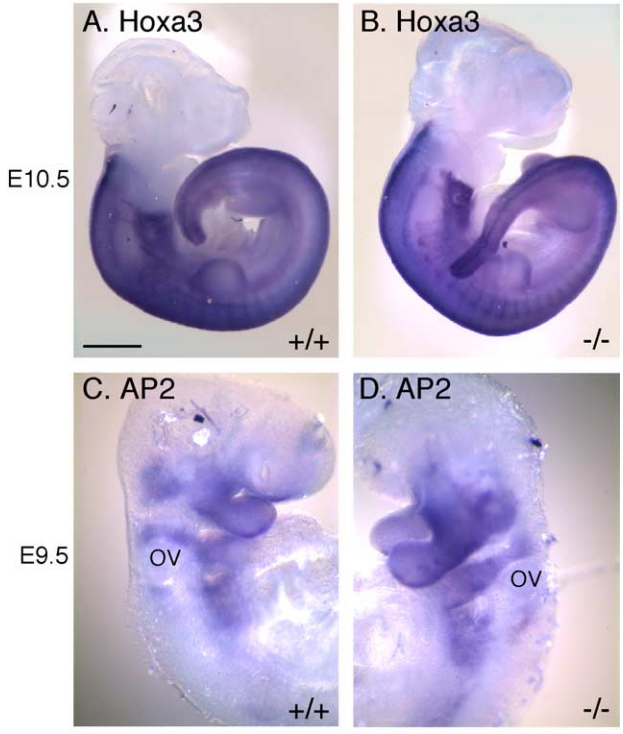

Fig. 5. *Hox*a3 and *AP2* expression are normal in *Pbx1*$^{-/-}$ embryos. Whole mount in situ hybridization for *Hoxa3* (A, B) at E10.5 and *AP2* (C, D) at E9 on wild-type and littermate *Pbx1*$^{-/-}$ embryos. ov, otic vesicle.

networks underlying these defects. As expected, *Hoxa3* expression was not changed at E9.5 or E10.5 (Figs. 5A, B, and data not shown), indicating that overall A–P patterning of the pharyngeal region was normal and underscoring the notion that Pbx works in parallel with Hox proteins. Neural crest migration also appeared relatively normal, as assayed by *AP2* expression in E9.0 *Pbx1*$^{-/-}$ embryos (Figs. 5C, D), and consistent with normal *Hoxa3* expression in NCC at E9.5 (data not shown).

Pax1 encodes a transcription factor that is present in all of the developing pharyngeal pouches, and serves as a marker for pouch formation. Accordingly, *Pax1* is expressed in the pouches from their initial formation through about E11.5 (Wallin et al., 1996). At E9.5 in control embryos, the first and second pouches were well formed, while the third pouch was just beginning to form (Fig. 6A). By E10.5, all four pharyngeal pouches were formed and expressed *Pax1*, with the third pouch showing the highest expression (Fig. 6C). In the *Pbx1*$^{-/-}$ embryos at E9.5 (24–26 somites), the first and second pouches appeared relatively normal (Fig. 6B). However, the third pouch was completely absent, as assessed both by morphology and by the absence of *Pax1* expression. By E10.5, a domain of *Pax1* expression appeared just caudal to the second pouch, although it did not have a morphology typical of the third pouch (Fig. 6D). These data suggested that third pouch formation was both delayed and abnormal in *Pbx1*$^{-/-}$ embryos.

We used additional molecular markers to further examine the patterning of the third pharyngeal pouches at the molecular level. *Gcm2* encodes a transcription factor required for parathyroid organogenesis, and is initially expressed in a highly restricted pattern corresponding to the putative parathyroid domain of the third pouch as early as E9.5 (Fig. 6E), and is subsequently maintained in the parathyroids during and after organ formation (Gordon et al., 2001). In E10.5 control embryos, *Gcm2* was expressed in a specific dorsal anterior domain of the third pouch (Fig. 6G). In the *Pbx1* mutant embryos, *Gcm2* was expressed in the third pouch at E9.75–E10 (28–30 somites), but at a lower level throughout the pouch compared to wild-type (Fig. 6F). This lower level, diffuse expression persisted through E10.5 in the mutants (Fig. 6H), in contrast to the strong localized expression in controls.

Mutation of *Tbx1*, a candidate gene for DiGeorge syndrome, results in complete deletion of the second, third,

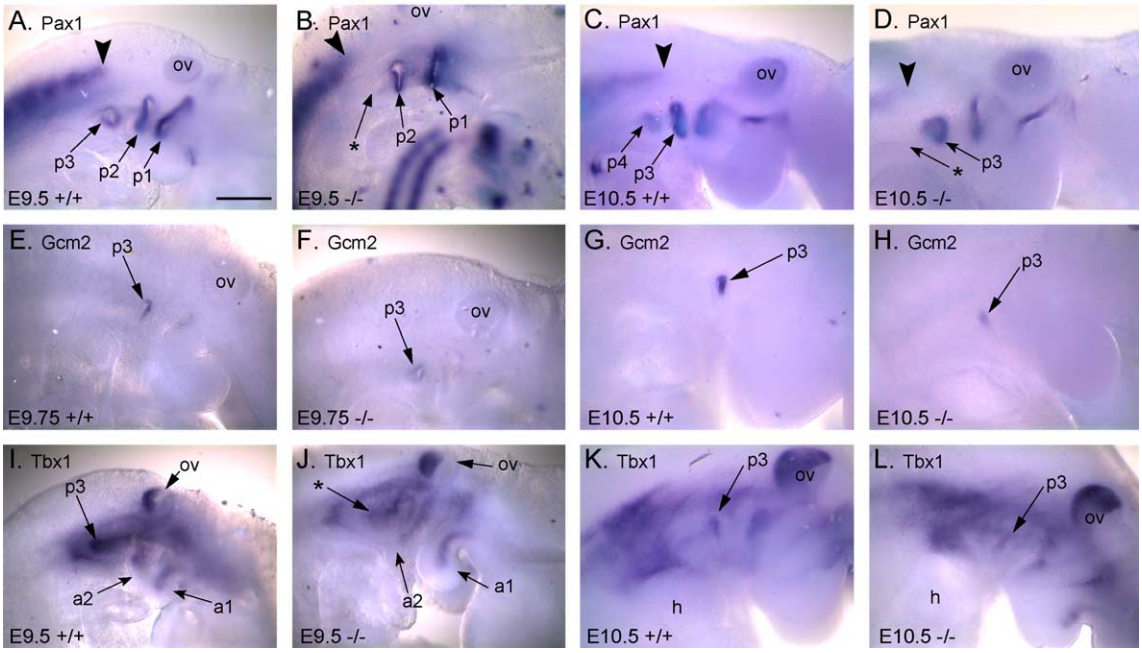

Fig. 6. Gene expression analysis of third pouch markers at E9.5 and E10.5 in *Pbx1*$^{-/-}$ embryos. Whole mount in situ hybridization for *Pax1* (A–D), *Gcm2* (E–H), and *Tbx1* (I–L) at E9.5–E10.5 on wild-type and littermate *Pbx1*$^{-/-}$ embryos. In all panels, anterior is to the right and dorsal is up. (A) At E9.5 *Pax1* is expressed in the first (p1), second (p2), and third (p3) pouches. Note the position of the third pouch relative to the most anterior somite staining for *Pax1* (arrowhead) and the otic vesicle (ov). (B) No *Pax1* staining is seen in the position where the third pouch should form (*) in an E9.5 *Pbx1*$^{-/-}$ embryo, although first and second pouch staining appears normal. (C) By E10.5, the fourth pouch is also positive for *Pax1*. (D) The third pouch is present at E10.5 in the *Pbx1* mutant, although its morphology appears abnormal compared to the wild-type control, but the fourth pouch is not present by *Pax1* staining. (E, G) At E9.75 and E10.5, *Gcm2* expression is seen only in the presumptive parathyroid anterior–dorsal domain. (F) In the *Pbx1*$^{-/-}$ embryo at E9.75, the third pouch has begun to form. However, *Gcm2* is expressed diffusely through the majority of the pouch. (H) At E10.5, the *Gcm2* expression domain is consistently reduced in intensity compared to controls, and is variable in its domain within the pouch. (I) Expression of *Tbx1* in a control embryo at E9.5 shows staining in the first and second arch mesodermal core, the posterior half of the otic vesicle, and the developing third pouch endoderm (p3). (J) In the E9.5 *Pbx1*$^{-/-}$ embryo, the only detectable change is loss of *Tbx1* in the region where the third pouch should form (*). (K) At E10.5, *Tbx1* expression in the third pouch is restricted to a specific anterior–dorsal domain, similar to the *Gcm2* domain shown in (G). (L) *Tbx1* expression in the third pouch of an E10.5 *Pbx1* mutant is more diffuse and spread throughout the pouch. h, heart.

and fourth pharyngeal pouches; not surprisingly, these mice are both athymic and aparathyroid (Jerome and Papaioannou, 2001). *Tbx1* is expressed in the pharyngeal pouch endoderm, peri-cardiac mesenchyme, and the mesodermal core of the first and second pharyngeal arches at E9.5–10.5 (Fig. 6I; (Garg et al., 2001). Strikingly, at E10.5 *Tbx1* expression within the third pouch endoderm closely resembled the highly localized *Gcm2* expression pattern in the dorsal third pouch endoderm (Fig. 6K). Most of the *Tbx1* expression pattern was normal in $Pbx1^{-/-}$ embryos. However, *Tbx1* expression in the third pharyngeal pouch was downregulated in $Pbx1^{-/-}$ mutants relative to controls in both E9.5 mutant embryos examined (Fig. 6J). By E10.5, the third pouch expression was consistently decreased in all three mutants examined, and more diffusely localized within the pouch (Fig. 6L) relative to the highly localized expression pattern in control embryos (Fig. 6K). The *Gcm2* expression pattern showed a similar change in the *Pbx1* mutants at this stage (Fig. 6H), suggesting that both *Gcm2* and *Tbx1* expression are similarly affected by the loss of *Pbx1*.

The E10.5 marker expression results suggested that the dorsal–ventral patterning of the third pouch was abnormal in the *Pbx1* mutants. However, the histological analysis showed that organ primordia were formed at E11.5, although they were very small. To determine whether these small third pouch primordia were patterned into thymus and parathyroid-specific domains, we used the *Gcm2* and *Foxn1* organ-specific markers. By E11.5 in control embryos, both *Gcm2* and *Foxn1* have well-defined complementary expression domains in the developing primordia, with *Foxn1* expressed in the large, ventral thymic domain (Fig. 7A) and *Gcm2* expressed in a much smaller, dorsal parathyroid domain (Fig. 7F) (Gordon et al., 2001). In the *Pbx1* mutants at this stage, *Foxn1* expression was absent either bilaterally (1/4) or on one side (2/4), and when present *Foxn1* expression was very low and in a much smaller domain relative to controls (Figs. 7B, C, E). In contrast, *Gcm2* expression was present in 3 of 3 *Pbx1* mutant embryos examined (Fig. 7G), and in one case was absent on one side (not shown). The *Gcm2* domains were consistently smaller than in controls (Fig. 7G), but the relative reduction of the *Gcm2* domain was not nearly as great as was seen with *Foxn1*. The *Foxn1* and *Gcm2* expression patterns suggest that the patterning of the primordia is abnormal in the *Pbx1* mutants. This result is consistent with the earlier relative expansion of the *Gcm2* expression pattern at E10.5, and suggests that the thymus-specific domain is selectively reduced and/or delayed in its development. Analysis of *Foxn1* expression at E12.5 showed that *Foxn1* is expressed at this stage in a more distinct pattern in the *Pbx1* mutants (Fig. 7E), although its domain is still reduced in *Pbx1* mutant embryos relative to controls (Fig. 7D), consistent with the smaller primordia seen in the histological analysis. These results were consistent with thymic epithelial specification and proliferation being delayed and possibly abnormal in the *Pbx1* mutants.

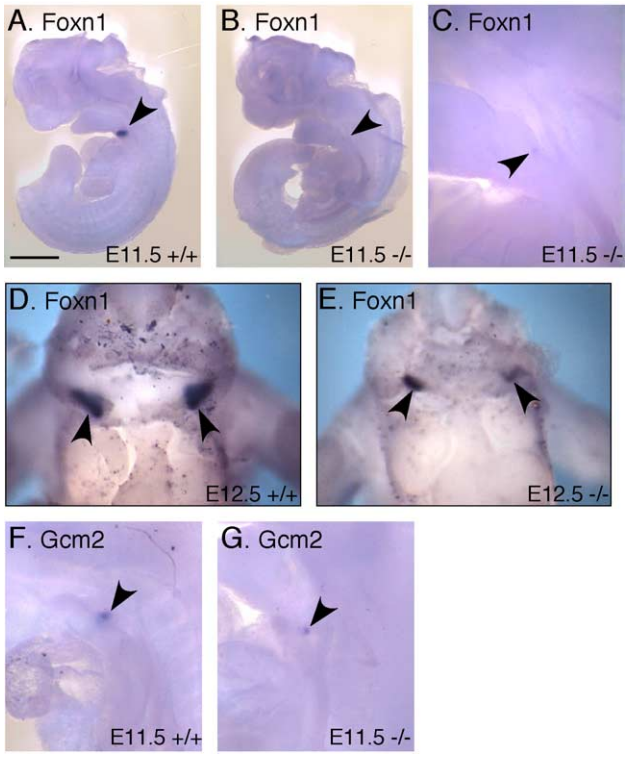

Fig. 7. Expression of thymus and parathyroid-specific markers is reduced and delayed in $Pbx1^{-/-}$ embryos. Whole mount in situ hybridization for *Foxn1* and *Gcm2* in E11.5–E12.5 control and $Pbx1^{-/-}$ embryos. A–C and F–G show the inside surfaces of E11.5 embryos hemisected in the sagittal plane. D–E show ventral views of partially dissected E12.5 embryos. In all panels, arrowheads indicate the common parathyroid-thymic primordia. (A) The thymic primordium at E11.5 expresses high levels of *Foxn1*. (B) An E11.5 $Pbx1^{-/-}$ embryo with an extremely small domain of *Foxn1* expression. (C) Higher magnification of the *Foxn1* expression domain in the embryo in (B). (D) Control embryo with bilateral, large thymic lobes expressing *Foxn1*. (E) $Pbx1^{-/-}$ littermate with bilateral hypoplastic *Foxn1*-positive thymic lobes. (F) *Gcm2* expression marks the parathyroid domain in an E11.5 wild-type embryo. (G) The *Gcm2* expression domain in a $Pbx1^{-/-}$ littermate is smaller than in the control. However, the reduction in the *Gcm2* expression domain is not as great as that seen in the *Foxn1* domain at the same stage (shown in B and C).

Discussion

We have presented evidence that *Pbx1* homozygous mutants have abnormal and delayed third and fourth pharyngeal pouch formation and patterning, resulting in defective or absent development of the pharyngeal pouch-derived thymus, parathyroids, and ultimobranchial bodies. After organ formation, proliferation of the thymic epithelium is decreased by 50%, resulting in further defects in organ development. These $Pbx1^{-/-}$ organ phenotypes are similar to phenotypes previously reported for *Hox1* and *Hox3* single and multiple gene mutants and to $Hoxa3^{+/-}Pax1^{-/-}$ compound mutants. There is also a variable effect on thyroid

development, which could be secondary to loss of the ultimobranchial bodies, consistent with the thyroid defect in *Hox*a3 mutants (Manley and Capecchi, 1995). Our results are consistent with Pbx1 acting together with multiple Hox proteins and in multiple cell types to regulate pharyngeal development.

At E9.5, when the caudal pharyngeal pouches are forming, Pbx1 nuclear localization was most prominent in the region where the fourth pouch will form, in the ectoderm, mesenchyme, and endoderm. This is consistent with the most severe and consistent phenotype in this region, the failure of fourth pouch formation. *Pbx1* mutants also displayed a delay in the formation of the third pharyngeal pouch at E9.5 as assayed by *Pax1* expression. These results further suggest that the dependence of *Pax1* expression on *Hoxa3* (Manley and Capecchi, 1995; Su et al., 2001) does not require *Pbx1*, since *Pax1* is expressed in *Pbx1* mutants, although delayed. The third pouch did form by E10.5; however, its patterning was abnormal as indicated by broadening of the *Tbx1* and *Gcm2* expression patterns from their normal, tightly defined dorsal domains. In contrast, NCC migration and A–P patterning of the pharyngeal region was normal, as demonstrated by marker analysis. These phenotypes in the third pouch correspond to a Pbx1 nuclear localization at E10.5 that is strongest in the pharyngeal ectoderm and endoderm, with more diffuse staining in the surrounding mesenchyme. Taken together, these results suggest that the *Pbx1* mutant phenotypes, particularly in pouch formation and patterning, may be endoderm-intrinsic.

The similarities in both the normal and mutant expression patterns of *Tbx1* and *Gcm2* in the third pouch are striking. We have previously shown that *Gcm2* expression is downstream of *Hoxa3* and *Pax1* (Su and Manley, 2000), and the current data further suggest that this regulation is at least in part Pbx1-dependent. Our data also suggest that *Tbx1* may also be downstream of Pbx1 function. Sequence analysis of the *Tbx1* promoter did reveal a *Pbx1* binding site, suggesting that the regulation of *Tbx1* by Pbx1 could be direct (L. Jerome and J. Papaioannou, personal communication). These results raise the possibility that *Tbx1* and *Gcm2* function in the same pathway downstream of *Pbx1* in caudal pharyngeal pouch development. Interestingly, both *Tbx1* and *Gcm2* expression have also been shown to be downstream of Shh signaling (Garg et al., 2001; Yamagishi et al., 2003). Alternatively, the similar changes in *Tbx1* and *Gcm2* expression detected in the *Pbx1* mutants could be an indirect effect of delayed and abnormal third pouch formation.

The defects in pouch formation and patterning in the *Pbx1* mutants are also reflected in the subsequent defects in pharyngeal organ formation. Consistent with the failure of fourth pouch formation, ultimobranchial bodies did not form. The third pouch-derived organogenesis phenotypes were somewhat less severe, as at least one organ primordium was present in all of the embryos we examined at E11.5–12.5, although when present were often very small. At these stages in control embryos, the third pouch-derived primordia are patterned into parathyroid- and thymus-specific domains by the expression of *Gcm2* and *Foxn1* (Gordon et al., 2001). In *Pbx1* mutants at E11.5, these small primordia usually expressed detectable levels of *Gcm2*, and in 50% of the mutant embryos examined also expressed *Foxn1*, indicating that they were patterned into organ-specific domains. Since *Foxn1* expression is normally initiated at E11.25 (Gordon et al., 2001), those mutant embryos in which *Foxn1* expression was absent at this stage either represent cases of absent primordia or could reflect a delay in organ development, consistent with the delayed third pouch formation at E10.5. The small organ primordia at these stages could also reflect an additional role for *Pbx1* in the mesenchyme during organogenesis, as the surrounding mesenchyme has been shown to provide growth signals for the developing primordium as early as E12.5 (reviewed in (Manley and Blackburn, 2003). This would be consistent with our previous work showing that Pbx1 is required for mesenchymal condensation and patterning during axial and appendicular skeletal development (Selleri et al., 2001).

These earlier defects in third pouch-derived organ formation result in three detectable phenotypes at later stages, and are associated with decreased proliferation in the developing primordium. In some cases, the thymus and parathyroids are absent, presumably due to failure of initial or sustained proliferation, as organ primordia were present in most cases at E11.5, but showed reduced or absent BrdU incorporation. In embryos in which the thymus was present, this reduced proliferation could account for the observed organ hypoplasia. The observed reduction in the thymic epithelial cells is similar to that previously reported in *Hoxa3*$^{+/-}$;*Pax1*$^{-/-}$ mutants (Su et al., 2001), suggesting that a Hoxa3–Pbx1 interaction may be involved in promoting early thymic epithelial cell proliferation. Of the thymic lobes that were present at E12.5 and later, most were ectopic. However, in contrast to the ectopia seen in *Hoxa3*$^{+/-}$;*Pax1*$^{-/-}$ mutants, the ectopic organs seen at later stages in *Pbx1* mutants were not due to delayed release from the pharynx, but appeared to be the result of a defect in the intrinsic ability of the organ primordia to migrate within the embryo.

As many of the functions for Pbx1 may be mediated by interactions with Hox proteins, comparison to *Hox* mutants with pharyngeal region phenotypes could help to identify potential partners for Pbx1. *Hoxa1;Hoxb1* double mutants have a loss of the second arch and pouch and second pouch-derived structures, and abnormal formation of the third pouch and deletion of thymus and parathyroids; a fourth pouch phenotype was not reported (Rossel and Capecchi, 1999). *Pbx1* mutants have a second pharyngeal arch, but it displays morphologic transformation of skeletal structures (Selleri et al., 2001) reminiscent of, but not identical to, the morphologic anteriorization observed in *Hoxa2* mutants (Rijli et al., 1993). Our data show that *Pbx1* mutants display

normal second pouch formation, but do have delayed and abnormal third pouch development, with some deletion of the thymus and parathyroids. Therefore, some phenotypes are similar, but less penetrant or less severe than in the *Hoxa1;Hoxb1* double mutants or *Hoxa2* single mutants. Strikingly, *Pbx1* mutants have defects in the same organs that are affected in *Hox3* single and multiple mutants. *Hoxa3* mutants have normal pouch formation and less severe fourth pouch phenotypes (organ formation is normal, but defects appear later in development) compared to the *Pbx1* mutants. In contrast, *Hoxa3* mutants display more severe 3rd pouch organ phenotypes (complete absence of thymus and parathyroids) (Chisaka and Capecchi, 1991; Manley and Capecchi, 1995). Interestingly, the *Pbx1* mutant phenotype most closely mimics the thymus and parathyroid phenotype of *Hoxa3;Hoxb3;Hoxd3* multiple mutants, which have ectopic and hypoplastic 3rd pouch-derived organs, although the embryonic origins of that phenotype are not known (Manley and Capecchi, 1998). This *Hox3* multiple mutant phenotype was proposed to be due to a neural crest mesenchymal defect, suggesting by comparison that the organ ectopia and hypoplasia seen in the *Pbx1* mutants could be due at least in part to interactions between Pbx1 and the Hox3 paralogous proteins in the mesenchyme. *Pbx1* and *Hoxa3* do not show any genetic interactions in double heterozygotes (NRM and LS, unpublished data), further suggesting that the pharyngeal organ defects may involve multiple *Hox3* paralogous genes.

Overall, the comparison of the *Pbx1* caudal pharyngeal pouch phenotype with known *Hox* mutant phenotypes reveals a diverse scenario. Some *Pbx1* phenotypes are strikingly similar to *Hox* phenotypes in this region, such as the organ ectopia and the partially penetrant loss of thymus and parathyroids, suggesting obvious partners for some Pbx1 functions. At the same time, other phenotypes are either more or less severe than *Hox* mutant phenotypes. The defects in pouch formation, particularly the fourth pouch, could be due to interactions between Pbx1 and multiple Hox1 and Hox3 proteins expressed in this region. Another interesting question is how some *Pbx1* single mutant phenotypes could be less severe than *Hox* phenotypes, since this could indicate that some Hox functions in this region might be Pbx1-independent. For example, the *Hoxa3* single mutant phenotype in the third pouch-derived organs is uniformly more severe than the *Pbx1* phenotype in the same region (Chisaka and Capecchi, 1991; Manley and Capecchi, 1995). This result could also indicate that some *Hox* gene functions in the pharyngeal region depend on interactions with other Pbx proteins. The two main candidates for providing redundant functions are Pbx2 and Pbx3. *Pbx2* is ubiquitously expressed during early development, but *Pbx2* single mutants have no detectable phenotype (Selleri et al., 2004). On the other hand, *Pbx3*-deficient mice do not have apparent defects in the pharyngeal region, but die in the early neonatal period from central respiratory failure due to abnormal activity of inspiratory neurons in the ventrolateral medulla (Rhee et al., in press). Analysis of compound *Pbx* gene mouse mutants will therefore be required to identify potential roles for other Pbx proteins in pharyngeal region development. A more intriguing possibility is that some Hox protein functions are completely Pbx-independent. A recent study generated a *Hoxb8* mouse mutant that was predicted not to bind Pbx proteins and yet resulted in unexpected dominant phenotypes (Medina-Martinez and Ramirez-Solis, 2003). Although much still remains to be understood about Hox–Pbx interactions and functions in vivo, our results unequivocally demonstrate that Pbx1 is required for caudal pharyngeal pouch development. Furthermore, our study indicates that mutation of *Pbx1* causes abnormalities in the caudal pharyngeal region that most closely mimic the phenotype of *Hox3* multiple mutants, consistent with a scenario where Pbx1 and a subset of Hox proteins act in concert to affect pharyngeal region development.

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