Pbx1 functions in distinct regulatory networks to pattern the great arteries and cardiac outflow tract

Ching-Pin Chang1,*,†, Kryn Stankunas1,*, Ching Shang1, Shih-Chu Kao1, Karen Y. Twu1 and Michael L. Cleary2,†

The patterning of the cardiovascular system into systemic and pulmonary circulations is a complex morphogenetic process, the failure of which results in clinically important congenital defects. This process involves extensive vascular remodeling and coordinated division of the cardiac outflow tract (OFT). We demonstrate that the homeodomain transcription factor Pbx1 orchestrates separate transcriptional pathways to control great-artery patterning and cardiac OFT septation in mice. Pbx1-null embryos display anomalous great arteries owing to a failure to establish the initial complement of branchial arch arteries in the caudal pharyngeal region. Pbx1 deficiency also results in the failure of cardiac OFT septation. Pbx1-null embryos lose a transient burst of Pax3 expression in premigratory cardiac neural crest cells (NCCs) that ultimately specifies cardiac NCC function for OFT development, but does not regulate NCC migration to the heart. We show that Pbx1 directly activates Pax3, leading to repression of its target gene Msx2 in NCCs. Compound Msx2/Pbx1-null embryos display significant rescue of cardiac septation, demonstrating that disruption of this Pbx1-Pax3-Msx2 regulatory pathway partially underlies the OFT defects in Pbx1-null mice. Conversely, the great-artery anomalies of compound Msx2/Pbx1-null embryos remain within the same spectrum as those of Pbx1-null embryos. Thus, Pbx1 makes a crucial contribution to distinct regulatory pathways in cardiovascular development.

KEY WORDS: Pbx, Hox, Pax3, Msx2, Heart development, Vascular patterning, Mouse

INTRODUCTION

Anomalies of the cardiac outflow tract (OFT) are among the most common congenital malformations in humans. They account for 20-30% of congenital heart anomalies (Sandler, 2004), which occur in at least 1% of live births and lead to significant morbidity and mortality. The high incidence of anomalies in part reflects the fact that cardiac OFT formation is a complex developmental process that requires several elaborate morphogenetic steps, including the division of a common arterial trunk, alignment of the divided arteries to their respective cardiac chambers, and the formation of valves for each arterial channel (Harvey and Rosenthal, 1999). Development of the great arteries that supply the head, neck and upper limbs is also a challenging task for developing embryos. This process involves extensive vascular remodeling of five pairs of primitive branchial arch arteries to form a distinctive arterial network. Perturbations of branchial arch artery patterning in humans result in a variety of vascular anomalies that often require surgical correction.

Development of the cardiac OFT and branchial arch arteries requires a specific subpopulation of neural crest cells (NCCs), the cardiac NCCs, which originate from rhombomeres 6, 7 and 8 in the hindbrain and migrate to the branchial arches and heart to regulate patterning of the branchial arch arteries and septation of the OFT, respectively (Kirby et al., 1983). Ablation of cardiac NCCs in the chick leads to characteristic cardiac and vascular anomalies, including persistent truncus arteriosus (PTA) and aberrant branchial artery patterning. Loss-of-function genetic experiments in mice have provided several models that recapitulate all or part of the NCC ablation phenotype in chick (reviewed by Kirby, 2007). Among a variety of signaling and transcriptional regulators, these studies have demonstrated crucial roles for several homeodomain transcription factors. Mice deficient for Hoxa3 have defects in branchial arch arteries consistent with a NCC defect (Chisaka and Capecci, 1991; Chisaka and Kameda, 2005; Kameda et al., 2003). Similarly, disrupted Hox expression in chick embryos is associated with abnormal patterning of the great arteries, but not with cardiac OFT defects (Kirby et al., 1997). With the exception of Hoxa3, however, single Hox gene deficiencies in mice have not been found to affect cardiovascular development, possibly reflecting redundancy in their contributions. Conversely, mutation of the Pax3 gene, which encodes a paired-homeodomain transcription factor, results in abnormal patterning of the branchial arch arteries and cardiac OFT (Conway et al., 1997; Epstein, 1996). Msx2, a homeodomain transcription factor, is an obligate repressed target of Pax3 in heart development (Kwang et al., 2002) as loss-of-function of Msx2 rescues the cardiac defects of Pax3 mutant mice.

Pbx1 is a TALE-class homeodomain transcription factor that forms heterodimeric complexes with a subset of Hox homeodomain proteins that are essential for regulating segmental identities during development (Chang et al., 1996; Chang et al., 1995; Knoepfler and Kamps, 1995; Peltenburg and Murre, 1996; Phelan et al., 1995). Interactions with Pbx1 confer a significant increase in the otherwise modest DNA-binding specificities and affinities of Hox proteins in vitro (Chang et al., 1996), and Pbx1 deficiency compromises Hox (Selleri et al., 2001) and para-Hox (Kim et al., 2002) protein functions in vivo. Pbx1 also partners with Meis/Prep proteins, members of the TALE class of homeodomain transcription factors (Abu-Shaar et al., 1999; Chang et al., 1997), which facilitate the formation of trimeric transcriptional complexes with Hox proteins (Jacobs et al., 1999). Consistent with the roles of Hox genes in specifying rhombomere identities, both Pbx and Meis orthologs regulate hindbrain development.
development in zebrafish (Choe et al., 2002; Waskiewicz et al., 2001; Waskiewicz et al., 2002). However, as the cardiac OFT in zebrafish does not normally divide into separate circulations, these previous studies did not address whether Pbx1 is required for the contribution of rhombomere-derived cardiac NCCs to OFT septation or branchial arch artery patterning.

In the current study, we demonstrate that Pbx1 impacts branchial arch artery patterning by controlling formation of the fourth and sixth branchial arches. Additionally, Pbx1 cooperates with Meis1 and/or Hox proteins to induce a high, but transient, activation of Pax3 in premigratory cardiac NCCs that ultimately governs their function, but not migration, during OFT septation.

MATERIALS AND METHODS
Mice
Pbx1−/−, Wnt1Cre and Pax3Cre mice have been described previously (Jiang et al., 2000; Li et al. and Pbx1 Cre, 2001). Pbx1+/− mice were maintained in a C57BL/6 background. Phenotypes of Pbx1-null mice were typically analyzed in embryos (E10.5-15.5) resulting from intercrossed parental mice that had been backcrossed to the C57BL/6 strain background for at least eight generations. Gestational age was determined by the date of observing a vaginal plug [set at embryonic day (E) 0.5], as confirmed by ultrasonography (Chang et al., 2003).

Angiography and vascular casting
Chest walls of mouse embryos were opened under microscopic visualization. A 33-gauge needle (Hamilton) mounted on a 1 ml tuberculin syringe was used to inject an acrylic resin (Batson no. 17) containing blue dye (Methyl Methacrylate Casting Kit, Polyscience) into the right ventricle. A syringe was used to inject an acrylic resin (Batson no. 17) containing blue dye into the right ventricle, while the embryos rested in saline (PBS). India ink (undiluted, water-insoluble form) was injected into the ventricles using a fine glass micropipette while the embryos rested in PBS. For India ink-based angiography, embryos were harvested at E10.5 or E11.5 and fixed overnight in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). For E11.5 embryos, the branchial arches and surrounding tissues were observed. Following angiography, embryos were held at 4°C for 2-6 hours to allow the resin to polymerize and cast the vasculature. Soft tissues of the embryos were subsequently dissolved in potassium hydroxide (Maceration Solution, Polysciences) at 55°C for 1-3 hours to expose the vascular casts, which were then cleaned and photographed under a dissecting microscope. For India ink-based angiography, embryos were harvested at E10.5 or E11.5 and fixed overnight in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). India ink (undiluted, water-insoluble form) was injected into the ventricles using a fine glass micropipette while the embryos rested in PBS. For E11.5 embryos, the branchial arches and surrounding tissues were carefully dissected to expose the branchial arch arteries prior to imaging.

Histology
Paraffin sections of mouse embryos were prepared as described previously (Stankunas et al., 2008a). Consecutive sections of 5-7 μm through the chest cavity were collected and stained with Hematoxylin and Eosin. RNA in situ hybridization and β-galactosidase staining
These procedures were performed as described previously (Stankunas et al., 2008a). The plexin A2, Pax3 and Msx2 antisense probes were as described previously (Brown et al., 2001; Kwang et al., 2002).

Immunostaining
Fluorescent immunostaining and immunohistochemistry on paraffin tissue sections (7 μm) were performed as previously described (Chang et al., 2004). The anti-Pbx1b monoclonal antibody (clone 41.1) (Chang et al., 1997) and anti-Pax3 monoclonal antibody (concentrate, Developmental Studies Hybridoma Bank) were used at 1:300 and 1:500 dilution, respectively, for immunostaining.

Electrophoretic mobility shift assay (EMSA)
EMSA was performed as described previously (Chang et al., 1995; Wu et al., 2007). Proteins (Pbx1a, Pbx2, Pax3, Meis1, HoxB2, HoxB4 and HoxB7) were prepared using a TnT Quick Coupled Transcription/Translation System (Promega) following the manufacturer’s instructions. Oligonucleotides probes corresponding to sequences from the Pax3 promoter were (5’ to 3’): Site A, CTCTACATCAAAAACGTCAAAGGCTT; Site B, CTCTCCTT-TTGATGTAAAGCTCT.

Luciferase reporter assays
The 1.6 kb promoter region of the Pax3 gene was amplified from mouse genomic DNA and cloned into the pGL3-basic vector (Promega). Expression plasmids for Pbx1b, Meis1 and HoxB4 were described previously (Chang et al., 1997; Chang et al., 1995). PC12 cells were co-transfected with expression plasmids, a Pax3 luciferase reporter construct and a constitutively expressing Renilla luciferase construct for normalization of transfection efficiency using Fugene 6 Transfection Reagent (Roche). Luciferase activities were analyzed using a Dual Luciferase Reporter Assay System (Promega). Fold activation was calculated relative to reporter baseline activities, and data presented as mean ± one s.d. P-values were determined using Student’s t-test.

RESULTS
Pbx1 is required for cardiac OFT septation
Septation of the cardiac OFT in mice occurs between E11.5 and E12.5, and the interventricular septum is sealed by E14.5 (Fig. 1A,D,F). By contrast, angiographic and serial histological analysis of Pbx1−/− embryos at E14.5 showed a single common arterial trunk (truncus arteriosus) that arose directly from the right ventricle with no arterial or OFT septation and no ductus arteriosus (Fig. 1B,E). The absence of the conal septum, which normally contributes to the development of the fourth and fifth arch arteries (Figs. 1C and 1F). In the current study, we demonstrate that Pbx1 impacts branchial arch artery patterning by controlling formation of the fourth and sixth branchial arches. Additionally, Pbx1 cooperates with Meis1 and/or Hox proteins to induce a high, but transient, activation of Pax3 in premigratory cardiac NCCs that ultimately governs their function, but not migration, during OFT septation.

Histology
Paraffin sections of mouse embryos were prepared as described previously (Stankunas et al., 2008a). Consecutive sections of 5-7 μm through the chest cavity were collected and stained with Hematoxylin and Eosin. RNA in situ hybridization and β-galactosidase staining
These procedures were performed as described previously (Stankunas et al., 2008a). The plexin A2, Pax3 and Msx2 antisense probes were as described previously (Brown et al., 2001; Kwang et al., 2002).

Immunostaining
Fluorescent immunostaining and immunohistochemistry on paraffin tissue sections (7 μm) were performed as previously described (Chang et al., 2004). The anti-Pbx1b monoclonal antibody (clone 41.1) (Chang et al., 1997) and anti-Pax3 monoclonal antibody (concentrate, Developmental Studies Hybridoma Bank) were used at 1:300 and 1:500 dilution, respectively, for immunostaining.

Electrophoretic mobility shift assay (EMSA)
EMSA was performed as described previously (Chang et al., 1995; Wu et al., 2007). Proteins (Pbx1a, Pbx2, Pax3, Meis1, HoxB2, HoxB4 and HoxB7) were prepared using a TnT Quick Coupled Transcription/Translation System (Promega) following the manufacturer’s instructions. Oligonucleotides probes corresponding to sequences from the Pax3 promoter were (5’ to 3’): Site A, CTCTACATCAAAAACGTCAAAGGCTT; Site B, CTCTCCTT-TTGATGTAAAGCTCT.

Luciferase reporter assays
The 1.6 kb promoter region of the Pax3 gene was amplified from mouse genomic DNA and cloned into the pGL3-basic vector (Promega). Expression plasmids for Pbx1b, Meis1 and HoxB4 were described previously (Chang et al., 1997; Chang et al., 1995). PC12 cells were co-transfected with expression plasmids, a Pax3 luciferase reporter construct and a constitutively expressing Renilla luciferase construct for normalization of transfection efficiency using Fugene 6 Transfection Reagent (Roche). Luciferase activities were analyzed using a Dual Luciferase Reporter Assay System (Promega). Fold activation was calculated relative to reporter baseline activities, and data presented as mean ± one s.d. P-values were determined using Student’s t-test.

RESULTS
Pbx1 is required for cardiac OFT septation
Septation of the cardiac OFT in mice occurs between E11.5 and E12.5, and the interventricular septum is sealed by E14.5 (Fig. 1A,D,F). By contrast, angiographic and serial histological analysis of Pbx1−/− embryos at E14.5 showed a single common arterial trunk (truncus arteriosus) that arose directly from the right ventricle with no arterial or OFT septation and no ductus arteriosus (Fig. 1B,E). The absence of the conal septum, which normally contributes to the sealing of the interventricular septum, resulted in a large, non-restrictive ventricular septal defect approaching the size of the normal aortic root (Fig. 1G).

Fig. 1. Pbx1 is required for cardiac OFT septation. (A-C) Angiographic casting of wild-type (A) and Pbx1+/− (B,C) E14.5 mouse embryos. (D-G) Hematoxylin and Eosin-stained transverse sections through the outflow tract (D,E) and ventricular septal regions (F,G) of wild-type (D,F) and Pbx1+/− (E,G) E14.5 embryos. The arrow indicates the interventricular septum. Ao, aorta; MPA, main pulmonary artery; RPA and LPA, right and left pulmonary arteries; PTA, persistent truncus arteriosus; RCA and LCA, right and left coronary arteries; DAO, descending aorta; VSD, ventricular septal defect.
The PTA in Pbx1−/− embryos displayed dual features of the aorta and main pulmonary artery in that it gave rise to coronary, pulmonary and systemic arteries. The right and left coronary arteries arose anteriorly, whereas a short stump of the main pulmonary artery arose posteriorly from the truncus (Fig. 1C). This short main pulmonary artery divided into the right and left pulmonary arteries (Fig. 1C), which were of similar size to wild-type pulmonary arteries (Fig. 1, compare A with C). After the coronary and pulmonary artery branching points, the truncus continued as the ascending aorta and generally arched to the left to form the descending aorta (Fig. 1B). These data demonstrate a requirement for Pbx1 in septation of the cardiac OFT.

Pbx1 regulates patterning of the branchial arch arteries

The aortic arch originates in the cervical region of the embryo during early development and, along with the developing heart, descends to its normal intrathoracic location by E13.5 (Fig. 2A). In Pbx1−/− embryos, the aortic arch failed to descend into the thorax and remained cervical in location (Fig. 2B), leading to a phenotype reminiscent of the human anomaly ‘cervical aortic arch’. As a consequence, the peripheral pulmonary arteries descended to the thorax to reach the lungs (Fig. 1C, Fig. 2E), in contrast to the normal course of pulmonary arteries within the thorax. In 18% (n=3/17) of Pbx1−/− embryos, the aorta arched to the right side, creating a mirror image of the arteries branching from the aorta (Fig. 2F).

The great arteries, which supply the head, neck and upper limbs, were aberrantly patterned in Pbx1−/− embryos (n=17). The left common carotid artery (CCA), which normally arises from the aortic arch, and the right CCA, which branches off the brachiocephalic artery (BCA), were generally absent in Pbx1−/− embryos (Fig. 2C,D). In the absence of the CCA, the external carotid artery (ECA) and internal carotid artery (ICA) arose directly from the aortic arch (Fig. 2D). Occasionally, a residual stump of CCA was present, connecting the ECA and ICA to the aortic arch (Fig. 2D and data not shown). The identity of the ECA was confirmed by its branching into facial and superficial temporal arteries (not shown); the ICA identity by its continued course into the cranium. The right subclavian artery (RSA), which normally arises from the BCA (Fig. 2C), originated instead from the descending aorta distal to the origin of the left subclavian artery (LSA) in Pbx1−/− embryos (Fig. 2E). The LSA, instead of arising from the aortic arch, arose from the descending aorta in Pbx1−/− embryos (Fig. 2C,E). The identities of the RSA and LSA were confirmed by their branching into internal vertebral (IVA), internal mammary (IMA) and axillary arteries (AA) (Fig. 2E), and by their destination in the right and left forelimbs, respectively.

Pbx1 is required for caudal branchial arch development

To examine whether the abnormal great-artery patterning seen at E14.5 in Pbx1−/− embryos reflected aberrant remodeling or a failure to establish the initial complement of branchial arches, India ink injections were used to mark the arterial systems of E10.5 and E11.5 embryos, prior to branchial arch artery regression. Instead of possessing three branchial arch arteries on each side (Fig. 2G), Pbx1−/− embryos (n=5) had only one or two patent branchial arch arteries (on both left and right sides) (Fig. 2H). When two arch arteries were present, the caudal-most artery was always narrow. The anatomic position of the aortic arches relative to the branchial arches was consistent with a failure to establish the sixth, and frequently the fourth, branchial arch arteries.

The arch arteries are derived from mesodermal cells of the branchial arches, and Pbx1−/− embryos have abnormalities in the development of the pharyngeal pouches of the caudal branchial arches (Manley et al., 2004). We therefore examined whether the
branchial arches formed normally in the absence of Pbx1 by using Msx2 whole-mount in situ hybridization to mark mesenchymal cells of the arches (MacKenzie et al., 1992). At E10.5, the pharyngeal groove separating arch 3 and 4 was absent in Pbx1−/− embryos (Fig. 2, J versus I), and the fourth branchial arch was smaller than normal and showed reduced Msx2 staining. Therefore, the great-artery patterning defects in Pbx1−/− embryos are at least in part due to a failure to develop a full set of branchial arches, which might also underlie the absence or reduction of organs derived from the caudal pharyngeal region (Manley et al., 2004).

**Pbx1 is widely present in cells regulating branchial arch artery and cardiac OFT development**

The OFT septation and great-artery patterning defects observed in Pbx1-deficient embryos are similar to those previously associated with defects in cardiac NCC migration and/or function (Kirby et al., 1983), suggesting a possible role in these processes. Alternatively, the OFT defects could represent a failure of secondary heart field (SHF)-derived cells to populate the OFT or aberrations in endocardial cushion development. The presence of Pbx1b, the major isoform of Pbx1 during development (Schnabel et al., 2001), was examined by immunostaining in each of these relevant tissues. At E8.75, Pbx1b was present in all cells of the hindbrain, including the premigratory NCCs at the extreme dorsal tip of the neural tube (the neuroectodermal junction) (Fig. 3A). By E9.5, Pbx1b was found in many, but not all, cells of the neural tube, including the premigratory NCCs. Pbx1b was also detected in many paraxial mesoderm cells flanking the neural tube at both E8.75 and E9.5 (Fig. 3A,B). In the septating OFT at E11.0, Pbx1b was abundant in vascular smooth muscle cells (Fig. 3C). Pbx1b was also present throughout the mesenchyme of the endocardial cushion, most likely in cells derived from both endocardial cells (by an epithelial-to-mesenchymal transformation) and NCCs (Fig. 3C). Whereas Pbx1b was decreased in cardiomyocytes of the OFT, it remained present at low levels in cushion endocardial cells (Fig. 3C). An immunofluorescence based-staining method for Pbx1b demonstrated its broad, but not ubiquitous, presence in E9.5 embryos (Fig. 3D-F). A higher magnification view of the region encompassing the E9.5 aortic sac and OFT revealed abundant Pbx1b in SHF cells entering the OFT and in the ectoderm, with lower Pbx1b levels in endocardial cells (Fig. 3G). Pbx1b was also found in many, but not all, endoderm-derived cells of the pharyngeal pouches and in mesenchymal cells throughout the embryo, including in the branchial arches (Fig. 3H). Pbx1b was present only in the endocardium (at low levels), among cells of the atria and left ventricle (Fig. 3I). Our staining results are consistent with other studies (Manley et al., 2004; Schnabel et al., 2001; Selleri et al., 2001; Stankunas et al., 2008) and indicate many possible sites of action for Pbx1b in branchial arch artery and OFT development, including cardiac NCCs.

**Pbx1 is not required for migration of cardiac NCCs into the outflow tract**

NCC migration was assessed by whole-mount RNA in situ hybridization with a plexin A2 probe that stains post-migratory NCCs (Brown et al., 2001). In Pbx1+/− embryos, plexin A2 staining highlighted two streams of NCCs migrating into the cardiac OFT, as seen in wild-type embryos (Fig. 4A,B). The absence of a generalized NCC migration defect in Pbx1+/− embryos was confirmed by plexin A2 staining of the dorsal root ganglia and sympathetic chains (Fig. 4C,D). Cell fate mapping, using Wnt1 promoter-driven Cre activity and the Rosa26Rcre line to mark NCCs and their derivatives (Jiang et al., 2000), showed that NCCs migrated into the cardiac OFT of both wild-type and Pbx1-null embryos (Fig. 4E,F). Thus, although Pbx1 is normally present in cardiac NCCs and their derivatives, its absence does not detectably affect their migration and appropriate localization to the cardiac OFT.

**Pbx1 is required for Pax3 promoter activity in rhombomeres where cardiac NCCs originate**

To further assess the impact of Pbx1 deficiency on NCCs, fate mapping studies were performed using Pax3Cre transgenic mice, which express Cre under the control of the Pax3 1.6 kb proximal promoter (Li et al., 2000). Like Wnt1Cre, Pax3Cre targets Cre activity to cardiac NCCs at their rhombomeric origins, prior to
delamination from the neural tube (Li et al., 2000). In marked contrast to wild-type embryos, Pax3 promoter-driven Cre activity was not detected in the cardiac OFT of Pax1<sup>+/−</sup> embryos at E12.5 (Fig. 4G,H), even though cardiac NCCs successfully migrated into the OFT (Fig. 4B,F). To determine whether the absence of Pax3Cre-marked cells in the cardiac OFT results from inactivity of the Pax3 promoter in premigratory cardiac NCCs, we examined the expression of β-galactosidase (lacZ) in the rhombomeres of E10.5 Pax3Cre;R26R<sup>lacZ</sup>;Pbx1<sup>−/−</sup> mice. In Pax1<sup>+/−</sup> embryos, Pax3Cre drove lacZ expression in rhombomere (R) 2, 4 and 6, and in the streams of NCCs migrating from these regions (Fig. 4K). By contrast, Pax3Cre activity in Pax1<sup>+/−</sup> embryos was selectively absent from R6 (Fig. 4L). Conversely, similar studies using the Wnt1Cre;R26R<sup>lacZ</sup> combination showed that Wnt1 promoter activity was maintained in cardiac NCCs from R6, R7 and R8 in Pax1<sup>+/−</sup> embryos (Fig. 4J). The absence of Pax3Cre and preservation of Wnt1Cre activity at the dorsal end of R6 was confirmed by histology of consecutive transverse sections in which R6 was marked by the caudal end of the otic vesicle (Fig. 4M-P). The difference between Wnt1Cre and Pax3Cre promoter activity in Pax1<sup>+/−</sup> embryos does not reflect earlier activity of the Pax3 promoter because, if any difference is present, Wnt1Cre activity initiated prior to that of Pax3Cre (see Fig. S1 in the supplementary material). Similarly, the Wnt1Cre domain in R6 through R8 appeared to entirely overlap with the missing R6 expression normally driven by the Pax3 promoter. Therefore, the absence of Pax3Cre-marked cells in the OFT of Pax1<sup>+/−</sup> embryos is not due to the loss of a subpopulation of Pax3-positive Wnt1-negative cardiac NCCs, but rather suggests a failure to activate the Pax3 promoter in premigratory cardiac NCCs.

**Pbx1 regulates transient expression of Pax3 in premigratory NCCs**

The loss of R6 expression driven by Pax3Cre but not Wnt1Cre implied that Pbx1 regulates transcription of the Pax3 1.6 kb proximal promoter, a possibility consistent with the requirement for a Pax3 binding site within the Pax3 proximal promoter for its full activity in embryos (Pruitt et al., 2004). However, using whole-mount in situ hybridization for Pax3 transcripts, we found no clear change in Pax3 in the neural tube of E9.5 Pax1<sup>−/−</sup> embryos (Fig. 5A). This surprising result led us to examine the presence of Pax3 in more detail using immunostaining of paraffin sections of wild-type and Pax1<sup>−/−</sup> null embryos. In an 18-somite wild-type embryo (equivalent to E8.75), Pax3 appeared in a broad dorsal band in the neural tube of R6 (Fig. 5B). Interestingly, this cluster of cells that strongly expresses Pax3, which corresponds to the premigratory neural crest, was absent in a littermate Pax1<sup>+/−</sup> embryo (Fig. 5C). However, Pax3 was still found in migrating NCCs in the absence of Pax1 (Fig. 5C). We examined the presence of Pax3 in the dorsal neural tube of the hindbrain from E8.0 through E9.5. Pax3 was detected in a broad dorsal band in the neural tube of a zero-somite (E8.0) embryo (Fig. 5D), a pattern that was retained throughout the developmental stages examined (Fig. 5E-G). By contrast, the robust Pax1-dependent presence of Pax3 in premigratory NCCs was found only transiently, between E8.5 and E9.0 (Fig. 5B,E,F). At E9.5, Pax3 was retained in a broad band of cells of the R6 dorsal neural tube, whereas the premigratory NCCs had relatively low levels of Pax3 protein (Fig. 5G). Taken
together, these results show that Pbx1 is required specifically for a transient burst of Pax3 expression in premigratory NCCs of R6 prior to their delamination from the neural tube.

**Pbx1 transcriptional complexes activate the Pax3 promoter**

In vitro studies were conducted to further assess the potential role of Pbx1 in the transcriptional regulation of Pax3, which contains Pbx1 binding sites in its promoter (Pruitt et al., 2004) (Fig. 6A). Electrophoretic mobility shift assays (EMSA) (Chang et al., 2004; Chang et al., 1995) confirmed that Site A, which contains a consensus Pbx1/Meis1 binding sequence (5’-TGACAGTT-3’) (Chang et al., 1997), supported robust cooperative binding by Pbx1 and Meis1, but not binding by either protein alone (Fig. 6B). By contrast, Pbx1 did not form binding complexes with several representative Hox proteins (HoxB2, HoxB4 or HoxB7) on Site A (Fig. 6B and data not shown). Site B, which is located 1.1 kb upstream of the Pax3 transcriptional start site, was bound robustly by HoxB4 or Meis1 in the presence of Pbx1 (Fig. 6C). DNA binding by HoxB2 and HoxB7 on Site B was also dependent on Pbx1 (data not shown). Pbx1-Meis1-Hox trimeric complexes did not form on either isolated Site A or Site B (Fig. 6B,C). The requirement for Pbx1 in regulating Pax3 promoter activity was assessed using a reporter gene containing the 1.6 kb proximal promoter driving luciferase expression and co-transfected plasmids expressing the indicated transcription factors. Fold activation was calculated relative to reporter baseline activity following normalization and is presented as the mean ± one s.d. P-values were determined using Student’s t-test. Whole-mount in situ hybridization for Msx2 (blue) in the hindbrain (dorsal view) in Pbx1+/+ (E) and Pbx1+/– (F) E9.5 embryos. The bracketed regions indicate R6-8. (G,H) Msx2 in situ hybridization (brown) on sections through R6 of Pbx1+/+ (G) and Pbx1+/– (H) E9.5 embryos. The sections are counterstained with Hematoxylin (blue). The arrows indicate premigratory neural crest. O, otic vesicle; R, rhombomere.

**Pax3 misexpression contributes to outflow tract defects in Pbx1+/– embryos**

Cardiac OFT defects in Pax3 mutant embryos arise from derepression of its downstream target gene Msx2, as demonstrated by rescue of the PTA in Pax3+/–;Msx2+/– embryos (Kwang et al., 2002). Since Msx2 is repressed by Pax3, the activation of which requires Pbx1, we examined Pbx1+/– embryos for misexpression of Msx2. By whole-mount RNA in situ hybridization, Msx2 transcripts were detected in the neural tube of E9.5 wild-type embryos (Kwang et al., 2002), with high levels in R5 and lower levels in the cardiac NCC-originating R6-R8 (Fig. 6E). By contrast, considerably higher
levels of Msx2 transcripts were detected in R6 through R8 of littermate Pbx1–/– embryos (Fig. 6F). RNA in situ hybridization of tissue sections at the R6 level confirmed enhanced expression of Msx2 transcripts in the dorsal neural tube in Pbx1–/– embryos (Fig. 6G,H). These results, which mirror those reported in Pax3–/– embryos, suggest that decreased Pax3 expression in Pbx1–/– embryos results in derepression of Msx2, which then causes the observed septation defects.

To assess whether Msx2 misexpression contributes to the developmental abnormalities in Pbx1–/– embryos, mice with compound deficiencies of Pbx1 and Msx2 were generated by interbreeding. Pbx1–/–;Msx2–/– embryos (Fig. 7D) were grossly similar to Pbx1–/– embryos (Fig. 7B); they showed edema and did not survive past E14.5, in contrast to the normal appearance of Msx2–/– embryos (Fig. 7A,C). Thus, increased rhombomeric Msx2 expression resulting from Pbx1 deficiency does not account for the gross defects observed in Pbx1–/– embryos. Furthermore, angiography revealed that Pbx1+/+;Msx2+/− and Pbx1–/–;Msx2–/− embryos exhibited the same spectrum of great-artery malformations as seen in Pbx1+/− embryos, including cervical aortic arch, aberrant carotid and subclavian arteries (data not shown). This indicates that Msx2 misexpression is not the main cause of the arterial patterning defects in Pbx1–/− mice.

The potential recovery of NCC contributions to OFT septation in Pbx1–/− embryos lacking Msx2 was assessed by examining consecutive histologic sections through the cardiac OFT. This revealed significant rescue of septation of the distal (truncal) portion of the OFT in Pbx1+/+;Msx2+/− embryos (Fig. 7H,K) (4/6 embryos; mean length of septation, 75.8 μm) and Pbx1+/−;Msx2+/− embryos (data not shown) (3/4; mean, 47.5 μm), as compared with littermate Pbx1+/− embryos (0/3; mean, 6.7 μm) (P<0.015). Since rescue did not extend to the proximal (conal) region of the OFT (Fig. 7I,L,M), these Pbx1+/−;Msx2+/− embryos had a milder form of PTA, arising from the right ventricle with an associated ventricular septal defect. By comparison, Msx2–/− embryos had no defects in OFT septation (Fig. 7E-G). Septation of the distal truncal region of the OFT is provided by the NCC-derived aorticopulmonary septal complex (Hutson and Kirby, 2007). Thus, Pbx1–/– embryos deficient for one or both Msx2 alleles had significant recovery of NCC function, with septation of the truncal, but not conal, region. These results demonstrate that dysfunction of the Pax3-Msx2 transcriptional hierarchy contributes to septation defects in Pbx1–/− embryos, although it does not entirely account for the role of Pbx1, suggesting that Pbx1 impacts additional pathways to regulate cardiac NCCs or other tissues contributing to OFT development.

**DISCUSSION**

Pbx1 is a global developmental regulator implicated in the formation of many organ systems (Brendolan et al., 2005; DiMartino et al., 2001; Manley et al., 2004; Schnabel et al., 2003a; Schnabel et al., 2003b; Zhang et al., 2006). However, the specific subordinate pathways that mediate its developmental contributions have not been extensively characterized. Our current studies extend the roles of Pbx1 to major morphogenetic events underlying the patterning of branchial arch arteries and formation of the cardiac OFT (Fig. 8). Vascular abnormalities in Pbx1–/− embryos include cervical aortic arch, right-sided aortic arch, and retroesophageal vascular ring, each
of which is frequently encountered in human patients (De la Cruz and Markwald, 1998; Sandler, 2004). The absence of cardiac OFT septation in Pbx1+/− embryos, resulting in PTA, is partially accounted for by loss of Pax3 expression in premigratory NCCs, culminating in a failure of aorticopulmonary septation. Interestingly, the cardiovascular defects in Pbx1+/− embryos, combined with craniofacial abnormalities (Selleri et al., 2001) and hypoplastic thymus, thyroid and parathyroid glands (Manley et al., 2004), resemble the anomalies observed in patients with DiGeorge syndrome (Epstein, 2001), which results from a deletion of chromosome 22q11 that includes TBX1, CRKL and other genes (Merscher et al., 2001; Moon et al., 2006). These features underscore the contributions of Pbx1 to the development of the caudal branchial arches and their derived organs, in addition to the cardiac OFT.

Failure of Pbx1+/− embryos to establish the caudal branchial arches might result in the absence of the fourth and sixth arch arteries, eventually producing an abnormal great-artery pattern. Loss of the fourth and sixth arch arteries in Pbx1−/− embryos accounts for the anomalous derivation of the aortic arch from the third branchial arch and for the absence of the ductus arteriosus, which is normally derived from the sixth arch artery. The consequently more-rostral location of the aortic arch prevents the heart from descending into the thoracic cavity, resulting in a cervical position of the aortic arch and the heart. The third branchial arch artery might not remodel as normal, owing to increased blood flow in the absence of the fourth arch artery, as hemodynamics have recently been shown to cooperate in branchial arch artery remodeling (Yashiro et al., 2007). The anomalous aortic arch derived from the third arch artery lies at the position where the ICA and ECA normally branch from the CCA, resulting in all four carotid arteries emerging from the aortic arch. The change in position of the RSA to an origin off the descending aorta is likewise explained by the absence of the right fourth arch artery, which normally connects the RSA to the BCA. The LSA appears more caudal in origin because the heart has moved rostrally to a cervical location in Pbx1−/− embryos.

Misregulation of Hox activity, which depends on Pbx function, might contribute to the arch artery defects in Pbx1−/− embryos as Hox genes are known to regulate branchial arch artery patterning. Hoxa3-null mice exhibit regression of the third arch artery (Kameda et al., 2003), and antisense targeted to Hox transcripts causes aberrant arch arteries in chick embryos (Kirby et al., 1997). Despite the evidence for a role of Pbx/Hox genes in branchial arch development, chemical targeting of Hox mRNAs in the chick was not accompanied by cardiac OFT defects (Kirby et al., 1997). Nor have studies of Hox-deficient mice shown cardiac malformations, as seen in Pbx1-deficient embryos. This is likely to reflect redundancy in the contributions of Hox genes, which is circumvented by the broader Hox compromise induced by Pbx1 deficiency. Further studies of Pbx1-deficient mice are likely to yield novel insights into the contributions of Pbx1 and Hox genes to various regulatory pathways in cardiac development that might not be apparent from studies of Hox-deficient mice.

Our studies showing the requirement of a Pbx1-Pax3-Msx2 pathway in cardiac development provide an additional example that Pbx and Pax genes act together to regulate organ development. Pbx proteins are known to regulate the expression of Pax6 during pancreatic development (Zhang et al., 2006). Here, we demonstrate that Pbx1 regulates Pax3 expression to control development of the cardiac OFT and involving the function of NCCs. Besides OFT defects, Splotch mice, which are deficient for Pax3, exhibit defects in thymus, thyroid, parathyroid and branchial arch artery development, resembling the malformations observed in Pbx1−/− embryos and chicks ablated for NCCs (Conway et al., 1997; Epstein, 1996; Franz, 1989; Kirby et al., 1983; Kwang et al., 2002; Li et al., 1999). Similarities in these NCC-derived organ defects between Splotch and Pbx1−/− mice suggest that Pax3 misregulation might underlie the phenotypes observed in Pbx1−/− embryos, including branchial arch artery defects. The arch artery defects, however, do not involve Msx2 because Msx2+− mutations fail to rescue the great-artery malformations of the Pbx1−/− embryos, despite the rescue of cardiac OFT development.
Cardiac OFT defects seen in Pax3 mutants arise from derepression of its downstream target gene, Msx2, in rhombomeres where cardiac NCCs originate. This was demonstrated by rescue of PTA in Pax3+/−,Msx2+/− embryos (Kwang et al., 2002). In Pbx1−/− embryos, we observed a significant reduction of Pax3 and enhancement of Msx2 gene expression in rhombomeres contributing to cardiac NCCs. These observations, together with DNA-binding, cellular transactivation and transgenic reporter assays, indicate that Pax3 is a direct in vivo transcriptional target of Pbx1, and establish a Pbx1-Pax3-Msx2 transcriptional cascade in heart development. Genetic support for this conclusion is provided by a significant rescue of aortoenteric septation in 70% of embryos containing both Pbx1 and Msx2 mutations (n=10), as evidenced by reduction of the PTA to milder conal defects, which we have never observed in Pbx1−/− embryos (n=28). Given that Pax3 and Msx2 function cell-autonomously in NCCs to regulate cardiac OFT development (Kwang et al., 2002; Li et al., 1999), our rescue experiments suggest that misregulation of the Pbx1-Pax3-Msx2 pathway in NCCs (Kwang et al., 2002; Li et al., 1999), our rescue experiments suggest that misregulation of the Pbx1-Pax3-Msx2 pathway in NCCs confers a developmental role for Pbx1 in cardiac NCCs. These observations, together with DNA-binding, cellular transactivation and transgenic reporter assays, indicate that Pax3 is a direct in vivo transcriptional target of Pbx1, and establish a Pbx1-Pax3-Msx2 transcriptional cascade in heart development. Genetic support for this conclusion is provided by a significant rescue of aortoenteric septation in 70% of embryos containing both Pbx1 and Msx2 mutations (n=10), as evidenced by reduction of the PTA to milder conal defects, which we have never observed in Pbx1−/− embryos (n=28). Given that Pax3 and Msx2 function cell-autonomously in NCCs to regulate cardiac OFT development (Kwang et al., 2002; Li et al., 1999), our rescue experiments suggest that misregulation of the Pbx1-Pax3-Msx2 pathway in NCCs confers a developmental role for Pbx1 in cardiac NCCs.


