Pbx4 is Required for the Temporal Onset of Zebrafish Myocardial Differentiation

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Abstract: Proper control of the temporal onset of cellular differentiation is critical for regulating cell lineage decisions and morphogenesis during development. Pbx homeodomain transcription factors have emerged as important regulators of cellular differentiation. We previously showed, by using antisense morpholino knockdown, that Pbx factors are needed for the timely activation of myocardial differentiation in zebrafish. In order to gain further insight into the roles of Pbx factors in heart development, we show here that zebrafish pbx4 mutant embryos exhibit delayed onset of myocardial differentiation, such as delayed activation of tnt2a expression in early cardiomyocytes in the anterior lateral plate mesoderm. We also observe delayed myocardial morphogenesis and dysmorphic patterning of the ventricle and atrium, consistent with our previous Pbx knock-down studies. In addition, we find that pbx4 mutant larvae have aberrant outflow tracts and defective expression of the proepicardial marker tbx18. Finally, we present evidence for Pbx expression in cardiomyocyte precursors as well as heterogeneous Pbx
expression among the pan-cytokeratin-expressing proepicardial cells near the developing ventricle. In summary, our data show that Pbx4 is required for the proper temporal activation of myocardial differentiation and establish a basis for studying additional roles of Pbx factors in heart development.

**Keywords:** cardiomyocyte; differentiation; myocardial morphogenesis; outflow tract; proepicardium; zebrafish; pbx4

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1. **Introduction**

Temporal control of cellular differentiation is essential for animal development. During cardiac development, timely activation of cardiomyocyte differentiation genes is needed for the proper morphogenetic formation of ventricular and atrial cardiac chambers [1,2]. In zebrafish cardiac development, cardiomyocyte differentiation is tightly linked with the morphogenetic movements needed for the bilateral cardiac primordia to undergo cell migration and fuse at the midline to form the heart tube [1,3–5]. Understanding the mechanisms behind cardiomyocyte differentiation are, thus, critical to understanding how the heart forms.

Recent studies have implicated Pbx homeodomain pioneer transcription factors and their Meis cofactor partners as critical regulators of heart development [6–11]. Pbx and Meis encode TALE (Three Amino acid Loop Extension)-class homeodomain-containing DNA-binding proteins. In mice and zebrafish, multiple Pbx genes are broadly expressed [12,13]. Well-characterized as cofactors for Hox proteins, Pbx/Meis also act as pioneer factors for Myod to promote skeletal muscle differentiation [14–16]. We previously showed, using antisense morpholinos (MOs) to knock down pbx2 and pbx4, that pbx2-MO;pbx4-MO zebrafish embryos have delayed activation of cardiomyocyte differentiation [8]. The delayed differentiation and migration of myocardial precursor cells leads to defective myocardial morphogenesis and chamber formation in pbx2-MO;pbx4-MO embryos [8]. Our studies on heart and skeletal muscle support a model whereby Pbx proteins provide competence to respond to cell-lineage transcription factors to direct cellular differentiation programs.

In mice, Pbx1/2/3 and Meis1 are required for outflow tract development [6,7]. These studies showed that Pbx proteins act in neural crest cells to promote Pax3 expression necessary for outflow tract development [7]. However, other studies have suggested roles for Pbx/Meis proteins within cardiomyocytes. We previously demonstrated that Pbx/Meis proteins can directly bind the promoter of the myocardial differentiation gene myl7 in vitro [8]. Postnatally in mice, Meis1 is expressed in cardiomyocytes and promotes cardiomyocyte cell-cycle arrest [11]. PBX/MEIS binding sites are enriched in open chromatin in cardiac progenitor cell culture models, and Pbx binding sites are also associated with Tbx5 binding sites [9,10,17]. In spite of these studies, the requirements for Pbx factors in heart development have not yet been fully addressed.

Here, we present evidence that zebrafish pbx4 mutant embryos display delayed onset of myocardial differentiation and morphogenesis, resembling the previously characterized zebrafish pbx2/4 morpholino-knockdown phenotype [8]. Furthermore, we also demonstrate that pbx4 is required for establishing a proper outflow tract and proepicardium, which gives rise to the epicardial mesothelium
surrounding the heart. Finally, we provide evidence of Pbx expression in cardiomyocyte precursors and of heterogeneous Pbx expression in pan-cytokeratin-expressing proepicardial cells near the ventricle. Taken together, our results provide further evidence that Pbx proteins promote myocardial differentiation and suggest multiple roles for Pbx proteins in heart development.

2. Experimental Section

2.1. Zebrafish Husbandry

All experiments involving live zebrafish (Danio rerio) were carried out in compliance with Seattle Children’s Research Institute IACUC guidelines. Zebrafish were raised and staged as previously described [18]. Staging time refers to hours post fertilization (hpf) at 28.5 °C. In some cases, embryos were raised for periods at 24 °C. For studies prior to 24 hpf, somite (s) number was used for staging, and mutant and control embryos were somite-stage matched. In some cases, embryos were incubated in 1-phenyl-2-thiourea to inhibit pigmentation [18]. The wild-type stock and genetic background used was AB. The pbx4\textsuperscript{b557} mutant strain was previously described and is likely a null allele [19]. pbx4\textsuperscript{b557} genotyping was performed using forward primer 5′ACTCGGCGGACTCTCGCAAGC3′ and reverse primer 5′GGCTCTCGTCGGTGATGGCCATGATCT3′. The genotyping PCR product is 128 base pairs, and digesting with XbaI yields a 98 base pair product from the mutant allele. The Tg(myh6:EGFP)\textsuperscript{s958}, Tg(myl7:EGFP)\textsuperscript{twu34}, and Tg(myl7:h2afva-mCherry)\textsuperscript{sd12} strains have been described [20–22].

2.2. Whole-mount RNA in Situ Hybridization

The following cDNA probes were used: krox20 (egr2b-Zebrafish Information Network) [23]; tnnt2a [24]; myl7 [25]; vmhc [25]; myh6 [26]; nppa [26]; gata4 [27]; gata5 [27]; hand2 [28]; elnb [29]; tbx18 (MGC:194980); and ltbp3 [30]. Whole-mount in situ hybridization colorimetric and fluorescent in situ staining was performed as previously described [15,31], with the following modifications. For colorimetric NBT/BCIP stained embryos, dimethylformamide was used prior to stepwise glycerol clearing to 80% glycerol in 1X PBS. Antisense RNA probes for either colorimetric or fluorescent in situ hybridization experiments were diluted into 5% dextran sulfate hybridization buffer. To reduce non-specific colorimetric staining at 48 hpf and later stages, we immediately processed 4% PFA/1X PBS-fixed embryos for de-pigmentation using 1 part 0.1% KOH (vol.): 1 part 1X PBS-0.1% Tween (vol.): 0.1 part 30% hydrogen peroxide (vol.) for 3–4 h at room temperature with gentle agitation. After 1 μg/mL proteinase K digestion and post-fixation steps, 48 hpf and later stages were then stored in pre-hybridization buffer at −20 °C overnight until further use. Following staining and imaging, tail clips from post-in situ hybridized embryos were lysed and genotyped for pbx4 as above.

2.3. Whole-mount Zebrafish Immunostaining and Cardiomyocyte Cell Counting

Whole-embryo immunostaining was performed with the following primary antibodies: anti-Pbx (1:100, rabbit antisera, [32]), MF20 (1:50, supernatant, Developmental Studies Hybridoma Bank, University of Iowa), anti-GFP (1:500, Roche #11814460001), anti-mCherry (1:500, Rockland Antibodies & Assays #600-401-P16), anti-GFP (1:500, Abcam ab13970), and pan-cytokeratin (1:100,
Sigma C2562). Secondary antibodies were goat anti-rabbit 568 (1:800, Life Technologies #A-11011), goat anti-rabbit 594 (1:500, Life Technologies #R37117), goat anti-chicken 488 (1:500, Life Technologies #A-11039), and goat anti-mouse 488 (1:800, Life Technologies #A-11029). Whole-mount immunostaining was performed as previously described [33]. For anti-Pbx staining at 48 hpf and for anti-pan-cytokeratin staining, Dent’s fixative (cold 80% methanol and 20% DMSO; [34]) was used with overnight fixation at 4 °C, and a modified whole-mount staining protocol was used [35].

Cardiomyocyte nuclei were counted using Tg(myl7:h2afvamCherry)d12 [21] in pbx4b557/- and control sibling embryos. 26 hpf embryos were immunostained and whole mounted in 100% glycerol. 48 hpf embryo hearts were removed after immunostaining via microdissection and mounted in 30% glycerol/70% PBS-0.1% Tween. Nuclei were counted using ImageJ software [36]. Cell number data were analyzed using a Student’s t-test (2-tailed, equal variance).

2.4. Fluorescent Confocal Microscopy and Stereoscope Imaging

Fluorescent confocal microscopy (Leica SP5) was used for examining embryos using a 20X air objective (NA = 0.7). In instances where longer working distances were necessary, fluorescently stained specimens were imaged with an upright Olympus fluorescent single photon confocal equipped with a 20X water-dipping objective (NA = 0.95). For 12 s and 16 s stage fluorescent RNA in situ, ImageJ was used for pairwise stitching of original optical stacks taken with 20X air objective (NA = 0.7), using the method previously described [37].

3. Results

3.1. Zebrafish Pbx4 Mutants Exhibit Defective Heart Function

Pöpperl et al. [19] previously described pbx4b557 mutant embryos as having a swollen pericardium and thin, weakly beating heart, and we showed that pbx2-MO;pbx4-MO embryos also have pericardial edema and decreased heart rate [8]. We confirmed that pbx4b557 mutant embryos have pericardial edema and exhibit blood pooled by the atrium (Figure 1B). We assessed heart rates and found that while control 50 hpf sibling embryos had 135 ± 11 beats/min (n = 14), pbx4b557 mutant embryos had 105 ± 14 beats/min (n = 13). Thus, zebrafish Pbx4 is needed for proper heart development and function, consistent with the previous studies [8,19].

Figure 1. Defective heart development in pbx4 mutant embryos. (A,B) Lateral views of live (A) control and (B) pbx4b557/- embryos at 50 h post fertilization (hpf). pbx4b557 mutant embryos show pericardial edema (arrow in B) and blood pooled near the atrium.
(arrowhead in B). a, atrium. For controls, \( n = 23 \). \( pbx4^{b557} \) mutant embryos (\( n = 20 \)) all show similar phenotypes as in (B). Anterior is to the left.

3.2. \( Pbx4 \) is Required for Proper Temporal Onset of Cardiac Muscle Differentiation

To determine whether \( Pbx4 \) is required for the initiation of myocardial differentiation, we examined the onset of expression of early myocardial differentiation genes in \( pbx4^{b557} \) mutant embryos. At the 12 somite (12 s) stage (15 hpf), cardiac troponin T type 2a (\( tnnt2a \)) is absent or very reduced in cardiomyocyte precursors in the anterior lateral plate mesoderm (ALPM) of \( pbx4^{b557} \)-/ embryos compared to control embryos (Figure 2A,B). By 16 s (17 hpf), \( tnnt2a \) expression is still reduced in \( pbx4^{b557} \)-/ embryos compared to controls (Figure 2C,D). Two other early myocardial differentiation genes, \( pan\)-myocardial \( myl7 \), and ventricular \( vmhc \) [25], are also absent or very reduced in the ALPM of \( pbx4^{b557} \)-/ embryos at 16 s (Figure 2E–H). At the time of fusion of bilateral cardiomyocyte populations at 21 s (19.5 hpf), the majority of \( pbx4^{b557} \)-/ embryos display delayed fusion of \( myl7 \)-expressing cardiac primordia (6/7 embryos not fused, Figure 2J), compared to controls (1/13 embryos not fused, Figure 2I). Similarly, \( pbx4^{b557} \)-/ embryos display non-fused domains of \( vmhc \)-expressing cells (6/7 embryos not fused, Figure 2L) compared to controls (2/18 not fused, Figure 2K). Finally, analysis of atrial \( myh6 \)-expressing cardiomyocyte precursors revealed \( pbx4^{b557} \)-/ embryos with non-fused and diminished expression (4/5 embryos not fused, Figure 2N) compared to controls (3/17 not fused, Figure 2M). To determine whether the delayed cardiac primordia midline fusion phenotype at 21 s was due to a myocardial morphogenesis defect and not a general developmental delay of \( pbx4^{b557} \)-/ embryos, we assayed these cardiomyocyte markers at 27 s (22.5 hpf). We find that \( pbx4^{b557} \)-/ embryos continue to display dysmorphic patterning of \( myl7 \), \( vmhc \), and \( myh6 \) expression domains compared to controls (Figure 2O–T). In particular, myocardial domains in 27 s \( pbx4^{b557} \)-/ embryos can remain not fused or show anterior rather than normal posterior fusion ([4]; Figure 2O–U). These data are consistent with a delayed initiation of myocardial differentiation and subsequent morphogenesis defect of cardiomyocyte primordia in \( pbx4^{b557} \)-/ embryos.

To further investigate the myocardial differentiation defects in \( pbx4^{b557} \)-/ embryos, we examined expression of \( nppa \), a myocardial differentiation gene whose expression is absent in \( pbx2\text{-}MO;pbx4\text{-}MO \) embryos [8]. \( nppa \) initially turns on at about 24 hpf in control embryos [8]. At 27 hpf, \( nppa \) expression is reduced in \( pbx4^{b557} \)-/ embryos (Figure 2V,W), although it does not appear as strongly reduced as was observed in \( pbx2\text{-}MO;pbx4\text{-}MO \) embryos [8]. Taken together, these results show that the myocardial differentiation phenotype in \( pbx4^{b557} \)-/ embryos is similar to, although possibly not as severe as, the myocardial differentiation phenotype we previously characterized in \( pbx2\text{-}MO;pbx4\text{-}MO \) embryos [8].
Figure 2. Delayed onset of cardiomyocyte differentiation genes in pbx4 mutant embryos. 
(A–T, V–W) RNA in situ expression of cardiomyocyte differentiation genes (A–D) tntt2a, 
Developmental stages are indicated. Embryos are shown in dorsal view, anterior towards 
the left. (A–H) Expression of the Pbx-dependent gene krox20, marking rhombomeres 3 and 5 
(r3, r5 in A) in the hindbrain, was included to distinguish between control and pbx4<sup>b557</sup>-/- 
mutants [12,19]. Expression of myod, which is expressed in a stripe in each somite plus 
two additional pre-somatic stripes [38], was included for somite staging. Arrows indicate 
ALPM expression domains of cardiomyocyte differentiation genes. (A,B) At 12 s, tntt2a is 
absent or reduced in pbx4<sup>b557</sup>-/- embryos (n = 8; 6/8 absent, 2/8 reduced) compared to 
controls (n = 30; all similar). (C,D) at 16 s, tntt2a is reduced in pbx4<sup>b557</sup>-/- embryos (n = 7) 
compared to controls (n = 28). (E,F) at 16 s, myl7 is absent or reduced in pbx4<sup>b557</sup>-/- 
embryos (n = 9; 3/9 absent, 6/9 reduced) compared to controls (n = 22). (G,H) at 16 s, 
vmhc is absent or reduced in pbx4<sup>b557</sup>-/- embryos (n = 6; 1/6 absent, 5/6 reduced) compared to 
controls (n = 10). (I–T) At 21 s and 27 s, myl7, vmhc, and myh6 show delayed fusion and 
abnormal patterning of expression domains in pbx4<sup>b557</sup>-/- embryos compared to controls. 
For (I–N), numbers of affected embryos are provided in the text. For (O–T), numbers of 
affected embryos are graphed in (U). Arrows indicate expression domains of cardiomyocyte 
differentiation genes. (U) Graph displaying percentage of embryos at 27 s with either 
normally patterned (blue), dysmorphic or unfused (green), or anteriorly fused (purple) cardiac
primordia. (V–W) At 27 hpf, nppa is reduced in pbx4b557/- embryos (n = 4, all similar reduced expression) compared to controls (n = 24). Arrows indicate myocardial expression domains.

3.3. Early Myocardial Specification Is Not Reduced in Pbx4b557/- Embryos

We previously showed in pbx2-MO;pbx4-MO embryos that, while myocardial differentiation is reduced, myocardial specification appears normal [8]. We, therefore, investigated early myocardial specification in pbx4b557/- embryos. We find that myocardial specification genes gata4, gata5, and hand2 are robustly expressed in pbx4b557/- embryos at 10 s (Figure 3). In particular, we observe an expanded domain of hand2 expression, similar to what we previously reported in pbx2-MO;pbx4-MO and hand2-MO embryos (Figure 3E,F) [8]. These results show that, in contrast to myocardial differentiation markers, myocardial specification markers do not show reduced expression in pbx4b557/- embryos.

Figure 3. Early cardiomyocyte specification does not appear reduced in pbx4 mutant embryos. (A–F) RNA in situ expression at the 10 s stage of cardiomyocyte specification genes (A–B) gata4, (C–D) gata5, and (E–F) hand2 in the anterior lateral plate mesoderm (ALPM) in (A,C,E) control and (B,D,F) pbx4b557/- embryos. Expression of the Pbx-dependent gene krox20, marking hindbrain rhombomeres 3 and 5 (r3, r5 in A), was included to distinguish between control and pbx4b557/- embryos [12,19]. Arrows indicate ALPM expression domains in control embryos. The junction between hand2-expressing ALPM and posterior lateral plate mesoderm domains is noted by arrowheads (E). For (A–D), n ≥ 10 for each marker in pbx4b557/- embryos and n ≥ 30 for each marker in control embryos, all with similar expression patterns. For (E–F), pbx4b557/- embryos display expanded hand2 expression (n = 14; 13/14 with expanded expression) compared to control embryos (n = 47; all similar). Embryos are shown in dorsal view, anterior towards the left.

3.4. Pbx4 is Required for Cardiac Chamber Morphogenesis, Proper Outflow Tract Development, and Proepicardial Development
Our previous analyses showed that pbx2-MO;pbx4-MO embryos have dysmorphic ventricular and atrial chambers [8]. We examined pbx4*b557/- embryos and found that they also had dysmorphic chambers (Figure 4A,B). In particular, we observe variable bulges in the ventricle (arrow in Figure 4B; also see Figure 4F,I,J). These dysmorphic chambers are similar to what we observed in pbx2-MO;pbx4-MO embryos and may be due, at least in part, to the delayed and abnormal heart tube morphogenesis (Figure 2) [8]. To assess the basis of this chamber dysmorphogenesis, we counted cardiomyocytes at two stages, 26 hpf and 48 hpf, using the transgenic strain Tg(myl7:h2afva-mCherry)*sd12, which labels all myocardial nuclei [21]. At 26 hpf, we find no significant difference between pbx4*b557/- embryos and control siblings (Figure 4C,D,G). At 48 hpf, we find that both ventricular and atrial chambers show increased numbers of cardiomyocytes in pbx4*b557/- embryos compared to controls (Figure 4E–G). We also find that patterning of the outflow tract is disrupted in pbx4*b557/- embryos (Figure 4H–J). The outflow tract defects show variability: the outflow tract smooth muscle marker elastinb (elnb; [29]) is reduced in some pbx4*b557/- embryos (Figure 4I) and appears expanded in some pbx4*b557/- embryos (Figure 4J). Furthermore, we find evidence that Pbx4 is needed for proepicardial development, because pbx4*b557/- embryos show loss of expression of tbx18, a key marker of the proepicardium (Figure 4K,L) [39,40]. While our findings do not directly reveal the cause of the chamber and outflow tract dysmorphogenesis, these results suggest that Pbx4 may have multiple roles in heart development: an early role in promoting myocardial differentiation and morphogenesis, and later roles in repressing myocardial differentiation and regulating outflow tract and proepicardial formation.

Defects in zebrafish outflow tract development and elnb expression have been linked with defects in the second heart field (SHF), a source of later-differentiating myocardial progenitors after the initial cardiac tube is formed [30,41–45]. In particular, loss of the SHF marker lubp3 leads to a reduced outflow tract and reduced elnb expression, while increased lubp3 expression, seen in zebrafish embryos lacking cadm4, correlates with an expanded outflow tract [30,44,46]. We find that expression of lubp3 is disrupted in pbx4*b557/- embryos (Figure 4M,N). However, in contrast to the varied patterns of elnb expression in pbx4*b557/- embryos (Figure 4H–J), we see a consistent, diffuse, defective lubp3 expression pattern that appears broader but also weaker than that in controls (Figure 4M,N). Thus, these findings suggest that, while there appear to be defects in the formation of the SHF in pbx4*b557/- embryos, it is not yet clear how these defects lead to the variable outflow tract defects in pbx4*b557/- embryos.
Figure 4. Cardiac chamber, outflow tract, and proepicardial development defects in \( pbx4 \) mutant embryos. (A,B) RNA in situ expression of cardiomyocyte differentiation gene \( myl7 \) (red) at 48 hpf in (A) control (\( n = 16 \)) and (B) \( pbx4^{b557/-} \) (\( n = 4 \)) embryos. V, ventricle. A, atrium. Arrow in (B) points to abnormal bulge in \( pbx4^{b557/-} \) ventricle. (C,D) Cardiomyocyte nuclei (magenta) in 26 hpf \( Tg(myl7:h2afvamCherry)^{ad12} \) (C) control (\( n = 13 \)) and (D) \( pbx4^{b557/-} \) (\( n = 9 \)) embryos. (E,F) Cardiomyocyte nuclei (magenta) in 48 hpf \( Tg(myl7:h2afvamCherry)^{ad12} \) (E) control (\( n = 9 \)) and (F) \( pbx4^{b557/-} \) (\( n = 7 \)) embryos. \( Tg(myh6:EGFP)^{s958} \) (green) is used to identify atrial cells. (G) Graph displaying cardiomyocyte nuclei count data at 26 hpf and 48 hpf. Error bars represent standard deviation. * \( P < 0.00003 \). (H,J) RNA in situ expression of outflow tract smooth muscle marker \( elnb \) (green) and \( myl7 \) (magenta) at 72 hpf in (H) control and (I) \( pbx4^{b557/-} \) embryos. In control embryos (\( n = 15 \)), \( elnb \) expression appears as a ring (H). In \( pbx4^{b557/-} \) embryos, \( elnb \) expression can appear reduced (arrow in I; 5/9 embryos) or expanded and
bifurcated (arrows in J; 4/9 embryos). (K,L) RNA in situ expression of tbx18 at 48 hpf in (K) control and (L) pbx4^{b557/-} embryos. tbx18 expression in pectoral fin (arrowhead) and proepicardial cells (arrow) is lost in pbx4^{b557/-} embryos (n = 9) compared to controls (n = 11), while facial expression (asterisk) is maintained. Hearts and embryos are shown in ventral view, anterior toward the top. (M,N) RNA in situ expression of lbtp3 at 24 hpf in (M) control and (N) pbx4^{b557/-} embryos. lbtp3 expression in second heart field domain (arrows) appears broader yet weaker in pbx4^{b557/-} embryos (n = 10, all weaker expression) compared to controls (n = 45, all similar), while notochord expression (arrowheads) is maintained. Embryos are shown in dorsal view, anterior towards the left.

3.5. Pbx Expression Domains Support Multiple Roles for Pbx Proteins in Heart Development

The expression of Pbx proteins in premigratory neural crest cells has been shown to contribute to the outflow tract defects observed in mouse Pbx1 mutants, and Pbx proteins are also expressed in myocardial cells in the mouse embryo outflow tract, but Pbx expression in cardiomyocyte precursors has not been fully addressed [6,7]. To examine Pbx expression, we used a Pbx antiserum that was raised against zebrafish Pbx4 and crossreacts with Pbxc [32]. At the 10 s stage, wild-type zebrafish embryos show nuclear Pbx expression in the ALPM and largely throughout the embryo (Figure 5A,B). At later stages, we used the Tg(myl7:EGFP)\textsuperscript{twu34} reporter to identify differentiated cardiomyocytes with nuclear Pbx expression at 21 s (19.5 hpf; Figure 5C,D) and 48 hpf (Figure 5G). While overall the Pbx staining appears predominantly nuclear, there may be some cytoplasmic Pbx expression as well (Figure 5D, for example). We find that, at 21 s, the Pbx immunostaining signal is largely absent from pbx4/- embryos (Figure 5E,F), confirming that the Pbx antibody is predominantly recognizing Pbx4. These findings support a role for Pbx proteins acting in early cardiomyocytes to promote their differentiation. We also find heterogeneous expression of Pbx in the pan-cytokeratin-expressing proepicardial cells at 48 hpf near the ventricle of the heart (Figure 5H,1) [47]. Taken together, these results provide evidence of Pbx expression in early cardiomyocyte precursors, in differentiated cardiomyocytes, and in spatially-restricted pan-cytokeratin-expressing regions, supporting multiple roles for Pbx proteins in heart development.

4. Discussion

4.1. Pbx Proteins Promote Early Myocardial Differentiation and Morphogenesis

Our study provides support for an early role for Pbx proteins in promoting myocardial differentiation by showing that pbx4 mutant embryos have delayed activation of myocardial differentiation (Figure 2) and that Pbx proteins are expressed in both cardiomyocyte precursors and differentiated cardiomyocytes (Figure 5). The delayed onset of cardiomyocyte differentiation markers and the delayed morphogenesis of myocardial cells during cardiac fusion and heart tube formation that we observe in pbx4 mutant embryos also provides further support that myocardial differentiation and morphogenesis are intimately linked [1,4,48,49]. Studies in mice and other model organisms have revealed varied roles for Pbx proteins in promoting and inhibiting cellular differentiation in many
developmental contexts [13,50]. Studies of Pbx functions in skeletal muscle have demonstrated the critical role of Pbx proteins in promoting skeletal muscle differentiation, in particular through acting as pioneer factors for Myod [14–16,51–54]. Our studies here not only demonstrate that Pbx proteins promote early myocardial differentiation, but also reveal that pbx4 appears to inhibit later myocardial differentiation. Even though we have shown that Pbx proteins can bind the zebrafish myl7 promoter [8], how Pbx4 activates or represses myocardial differentiation genes at different stages of development is not yet known.

It is not yet clear whether the role of Pbx proteins in promoting early myocardial differentiation is conserved. Previous studies in mouse embryos, using combinations of Pbx gene null alleles, have shown that Pbx genes are required for outflow tract development [6,7], and we show that zebrafish pbx4 is needed for outflow tract development (Figure 4). However, because of early lethality of certain allelic combinations, the full requirements for Pbx proteins in mouse heart development have not yet been determined. Thus, studies have not yet directly addressed whether early myocardial differentiation is affected in Pbx-deficient mice.

Figure 5. Analysis of Pbx expression during zebrafish cardiac development. (A–G) Pbx immunostaining at (A–B) 10 s (14 hpf), (C–F) 21 s (19.5 hpf), and (G–H) 48 hpf. (A–B) Pbx expression (magenta) in wild type 10 s embryo co-stained with Hoechst to label nuclei (green). (A) Dorsal view, anterior to the left. The line in (A), approximately at the level of hindbrain rhombomere 3, shows where we took an optical cross-section view for (B).
4.2. Pbx4-dependent Patterning of the Developing Outflow Tract

Previous studies have shown that loss of different combinations of Pbx genes in mouse embryos can lead to a spectrum of outflow tract defects [6,7]. These studies in mouse embryos also demonstrated that Pbx proteins are expressed in many cell types involved in outflow tract development. In particular, Pbx1 expression in pre-migratory cardiac neural crest cells directly activates expression of Pax3, which is required for cardiac neural crest cells to properly contribute to outflow tract development [7,61]. Pbx proteins are also expressed in vascular smooth muscle cells of the outflow tract and great arteries in mouse embryos [6,7]. We find that zebrafish pbx4 is required for proper patterning of the smooth muscle elnb-
expressing domain of the outflow tract (Figure 4). In contrast to the loss of *elnb* expression in the outflow tract observed in loss-of-function studies for zebrafish *ltbp3*, *nkx2.5*, and *tbx1* [30,44,45], we observed a variable mispatterning of the *elnb* domain along the outflow tract in *pbx4* mutants (Figure 4). Loss of zebrafish *cadm4* function leads to an expansion of the outflow tract, although *elnb* expression was not examined [46]. In future studies, it will be important to determine whether *pbx4* is acting upstream or downstream of *tbx1* and these other factors involved in outflow tract development.

Our results point to two potential mechanisms by which *pbx4* might regulate outflow tract development. First, we observe an expansion of *hand2* expression in the ALPM of *pbx4* mutants (Figure 3). Early SHF cells arise from *hand2*-expressing cells in the early ALPM [41,44]. The expanded *hand2* expression domain in *pbx4* mutants could, thus, provide a possible source of additional SHF cells and, indeed, our results with *ltbp3* expression indicate a possible expanded SHF (Figure 4). An expanded SHF could lead to the increased cardiomyocytes that we observe at 48 hpf as well as the expanded *elnb* expression that we observe in some *pbx4* mutant embryos (Figure 4). Second, the opposite effects that we observe in *pbx4* mutants on early versus later cardiomyocyte differentiation is similar to the dual effects of bone morphogenetic protein (BMP) signaling on early versus later cardiomyocyte differentiation, and BMP signaling regulates outflow tract formation [41,62,63]. In future studies, it will be important to examine how *pbx4* regulates *hand2* expression and interacts with BMP signaling.

A recent study has found that Pbx1 directly regulates PDGFRβ expression in vascular smooth muscle progenitors in mouse kidney development [64]. PDGFRβ expression is also required for coronary vascular smooth muscle development and for cardiac neural crest cell contributions to outflow tract development in mouse embryos [65–67]. Whether PDGFRβ regulation is conserved in zebrafish and contributes to the phenotypes we observe in *pbx4* mutants remains to be determined. Genetic mosaic studies in zebrafish embryos should help establish in which cell types Pbx4 functions to regulate outflow tract development. Studies implicating Pbx proteins in outflow tract malformations in human congenital heart defects further underscore the importance of understanding how Pbx proteins function in heart development [68].

### 4.3. Pbx Expression Heterogeneity among Pan-Cytokeratin-Expressing Cells Adjacent to the Ventricle

Previous studies have demonstrated broad expression of Pbx proteins in many cell types involved in heart development [6,7]. Our zebrafish Pbx immunostaining data is in agreement with these studies and other previous reports of broad Pbx expression (Figure 5) [13,19]. While overall our Pbx immunostaining appears predominantly nuclear, there may also be some cytoplasmic Pbx expression (Figure 5). Nuclear and cytoplasmic expression of Pbx would be consistent with earlier studies showing that Pbx protein localization is regulated during development [69,70]. We were able to make use of pan-cytokeratin expression, a well-established marker of the developing epicardium in many species [39,71,72], to ask whether Pbx proteins are expressed in developing zebrafish proepicardial cells. We noticed that there is a subpopulation of pan-cytokeratin-expressing cells near the ventricle at 48 hpf that is devoid of nuclear Pbx expression (Figure 5). We also noticed a loss of *tbx18* expression in the developing proepicardial region in *pbx4*+/− embryos at 48 hpf (Figure 4). The proepicardium gives rise to the epicardium, a mesothelial lining of the heart that is needed for proper heart and
coronary vessel development and that has been implicated in cardiac regeneration [73–75]. The heterogeneity of Pbx expression among pan-cytokeratin expressing cells near the ventricle thus offers a platform for investigating potential actions of Pbx in proepicardial and epicardial cells during cardiac development and regeneration. Whether reduced tbx18 expression (Figure 4) represents the loss of an epicardial subset, or indicates tbx18 transcription is directly regulated by Pbx proteins, requires further work. What roles Pbx expression heterogeneity among cytokeratin-positive cells may play in the development and diversity of proepicardial cell fates also remains to be elucidated in future studies.

5. Conclusions

We show that zebrafish pbx4 mutant embryos exhibit delayed onset of myocardial differentiation, delayed myocardial morphogenesis, and dysmorphic patterning of the ventricle and atrium, consistent with our previous Pbx knock-down studies. In addition, we find that pbx4 mutant larvae have aberrant outflow tracts and defective expression of the proepicardial marker tbx18. We also present evidence for Pbx expression in cardiomyocyte precursors as well as heterogeneous Pbx expression among the pan-cytokeratin-expressing proepicardial cells near the developing ventricle. In conclusion, our data show that Pbx4 is required for the proper temporal activation of myocardial differentiation and establish a basis for studying additional roles of Pbx factors in heart development.
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Author Contributions


Conflicts of Interest

The authors declare no conflict of interest.

References


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