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Review

# Anterior *Hox* Genes in Cardiac Development and Great Artery Patterning

Brigitte Laforest \*, Nicolas Bertrand and Stéphane Zaffran \*

Aix Marseille Université, Inserm GMGF UMR\_S910, Faculté de Médecine, 27 Bd Jean Moulin, 13005 Marseille, France; E-Mail: nicolas.bertrand@univ-amu.fr

\* Authors to whom correspondence should be addressed; E-Mails: brigitte.laforest@univ-amu.fr (B.L.); stephane.zaffran@univ-amu.fr (S.Z.); Tel.: +33-4-91-324-936; Fax: +33-4-91-722-972.

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Abstract: During early development, the heart tube grows by progressive addition of progenitor cells to the arterial and venous poles. These cardiac progenitor cells, originally identified in 2001, are located in the splanchnic mesoderm in a region termed the second heart field (SHF). Since its discovery, our view of heart development has been refined and it is well established that perturbation in the addition of SHF cells results in a spectrum of congenital heart defects. We have previously shown that anterior *Hox* genes, including *Hoxb1*, *Hoxa1* and *Hoxa3*, are expressed in distinct subdomains of the SHF that contribute to atrial and subpulmonary myocardium. It is well known that *Hox* proteins exert their function through interaction with members of the TALE family, including Pbx and Meis factors. The expression profile of Pbx and Meis factors overlaps with that of anterior Hox factors in the embryonic heart, and recent data suggest that they may interact together during cardiac development. This review aims to bring together recent findings in vertebrates that strongly suggest an important function for Hox, Pbx and Meis factors in heart development and disease.

**Keywords:** *Hox*; heart development; great arteries; TALE; second heart field; neural crest cells; *Pbx*; *Meis* 

### 1. Introduction

Congenital heart defects (CHDs) occur in 1–2% of live births and lead to significant mortality and morbidity [1]. Anomalies in cardiac outflow tract (OFT) development are among the most common CHDs with a prevalence of 30%, likely reflecting the complex morphogenetic events underlying heart development. In the early embryo, cardiac progenitors are located in bilaterally paired heart fields in the splanchnic mesoderm of the anterior lateral mesoderm. At embryonic (E) day 7.5 in the mouse embryo, these cells form a crescent shape, also referred to as the first heart field (FHF), where differentiated cardiomyocytes are now observed (Figure 1). By E8.0, these cells merge along the ventral midline to form a primitive heart tube, containing endocardial cells at its interior layer and myocardial cells at its exterior layer. Subsequently, the forming heart tube undergoes rightward looping and begins to beat. Concomitant with cardiac looping, the heart tube increases dramatically in length by addition of myocardial cells at its arterial and venous poles. The population of cardiac progenitors located in the splanchnic mesoderm that contribute to this growth is termed the second heart field (SHF) [2,3]. Evidence for the contribution of SHF progenitor cells to the heart was revealed by three independent studies that identified a progenitor cell population in the pharyngeal mesoderm that contributes to the elongation of the embryonic OFT (see [3]). The SHF is marked by the expression of different transcription factors including *Islet 1 (Isl1)* [4], *Nkx2-5* [5] and *T-box 1 (Tbx1)* [6]. Since its discovery, there has been accumulating evidence showing that the SHF is pre-patterned along the anterior-posterior axis [7,8]. Briefly, the anterior part of the SHF, which is marked by Fgf10 expression, contributes to the formation of OFT and right ventricular myocardium whereas the posterior region of the SHF, which expresses *Hoxb1* and *Hoxa1*, contributes to a large part of the atrial and sub-pulmonary myocardium [4,7,9] (Figure 1). Fate mapping experiments recently revealed that the posterior SHF contributes not only to the inflow but also to the arterial pole, as observed by the presence of both *Fgf10*-expressing and non-expressing cells in the OFT of injected embryos [10].

The SHF cells are characterized by a proliferating, non-differentiated state. As SHF progenitor cells are added to the elongating heart tube, they are exposed to a number of signals or cues emanating from surrounding cell types, including pharyngeal ectoderm and endoderm [3,11]. Hence, a slight perturbation in the addition of SHF cells may result in a spectrum of conotruncal defects ranging from OFT misalignment to septation defects [12]. Increasing knowledge has been gained in the last couple of years about the signaling pathways playing critical roles in SHF deployment, and they have been reviewed in detail elsewhere [3,11]. During progressive heart tube elongation, SHF cells are submitted to pro-proliferative FGF, canonical Wnt and Hedgehog signals in the pharyngeal region. Following proliferation within the SHF, progenitor cells will be recruited to the arterial and venous poles where they are now exposed to signals from the BMP, non-canonical Wnt and Notch pathways, which positively regulate their differentiation. Studies in mouse and avian have linked retinoic acid (RA) signaling to anterior-posterior patterning of the heart tube [13-15]. Knockout mice deficient in retinaldehyde dehydrogenase 2 (Raldh2), which catalyzes the second oxidative step in RA biosynthesis, display abnormal hearts, with a highly hypoplastic inflow tract region [16]. More recently, two studies demonstrated that expression of Isl1, along with other SHF markers including Tbx1, Fgf8 and Fgf10 is caudally expanded in Raldh2<sup>-/-</sup> embryos [17,18]. Despite expansion of anterior SHF markers, cardiac differentiation was blocked in Raldh2 mutants, resulting in failure of

heart tube elongation [17]. In the *zebrafish*, RA signaling has also been shown to regulate the size of the cardiac field through indirect regulation of *hoxb5b* expression in the adjacent forelimb field [19]. Recently, cardiac enhancers containing RA-responsive elements have been identified and characterized in the *Hoxb1*, *Hoxa1* and *Hoxa3* genes [20,21]. Our lab has reported that manipulation of the RA signaling pathway actually shifts the rostral border of *Hoxa1*, *Hoxb1* and *Hoxa3* expression domains and lineage contribution to the heart [9]. Together, these findings suggest that *Hox* genes are sensitive to RA dosage and that influence of RA on heart development may be mediated through its effects on *Hox* genes.

**Figure 1.** Cardiac contribution of progenitor cells expressing *Hox* genes in the second heart field. Diagram showing stages of heart development and contribution of the first heart field (red) and second heart field (green) to the heart. Frontal view is shown for embryonic day 7.5 (E7.5), E10.5, E14.5 and lateral view for E8. As second heart field (SHF) progenitor cells are added to the elongating heart tube, they contribute to formation of the right ventricle, outflow tract (OFT) and both atria. The left ventricle is derived exclusively from the first heart field (FHF), which also contributes to atria formation. In the early embryo (E7.5–E8), *Hoxa1/b1/a3* expressing cells characterize distinct subdomains along the anterior-posterior axis of the SHF. At later stages, *Hoxa1/b1/a3* progeny contribute to both atria and the inferior wall of the OFT (E10.5), which subsequently gives rise to the subpulmonary myocardium (E14.5). AHF, anterior heart field; Ao, aorta; CC, cardiac crescent; cNCC, cardiac neural crest cells; Epi, epicardium; LA, left atrium; LV, left ventricle; PA, pharyngeal arch; Pt, pulmonary trunk; RA, right atrium; RV, right ventricle; pSHF, posterior second heart field.



Hoxb1 + Hoxa1 + Hoxa3-Cre lineages

Nearly 30 years ago, cardiac neural crest cells (NCC), which constitute a subpopulation of cranial NCC, were described as important for normal heart development [22]. Much of what we know today about the contribution and function of cardiac NCCs in heart development has been learned by studying migration and ablation of premigratory NCCs in chick embryos and quail-chick chimeras [23,24]. Cardiac NCCs migrate into the pharyngeal arches 3, 4 and 6 where they participate to the remodeling of the pharvngeal arch arteries. During heart development, cardiac NCCs also contribute to septation of the OFT and development of the conduction system. In addition, it was shown recently that preotic NCCs migrate beyond the conotruncal region of the heart, where they give rise to coronary smooth muscle cells and mesenchymal like cells in the IVS and papillary muscle of the RV [25]. Many signaling factors, including the BMP/TGFB, endothelin and PDGF signaling pathways, have been involved in specifying and triggering the migration of cardiac NCCs [23,26]. One of the defects observed following NCC ablation is abnormal cardiac looping, which results from failure of addition of SHF cells to the elongating heart tube. Instead of migrating and subsequently differentiating into myocardium, SHF cells kept proliferating, suggesting that a specific factor is regulated by NCCs in the pharynx. Recently, it was shown that FGF8 signaling is elevated in the pharynx of NCC-ablated embryo concomitant with cardiac looping, likely indicating that FGF8 could be the potential factor [27]. Overall, these findings reveal a complex relationship between cardiac NCCs and the SHF and any perturbation in the interaction between these two cell types may lead to conotruncal heart defects.

Hox genes form a large family of evolutionarily conserved homeodomain transcription factors that regulate positional identity along the anterior-posterior axis [28,29]. In mammals, Hox genes are organized into four different clusters and are expressed in defined and often overlapping domains along the body axis in a manner corresponding to their position along the chromosome (temporal and spatial collinearity) [30]. Consistent with this, disruption of *Hox* gene function frequently results in the transformation of one specific structure into another, the so-called homeotic transformation. Hox gene expression in the mammalian heart was first characterized in the chick, where expression of Hoxa4, Hoxd3 and Hoxd4 was detected at early stages of cardiogenesis, which was further increased upon RA treatment [31]. Using co-staining experiments, our lab has reported that *Hoxa1*, *Hoxb1* and *Hoxa3* are expressed in distinct subdomains in the SHF that contribute to atria and OFT region but they were never detected in the heart tube nor the right ventricle [9]. In addition, lineage tracing experiments showed that cells that have expressed *Hoxa1*, *Hoxb1* and *Hoxa3* contribute not only to the inflow but also to the arterial pole, more specifically to the OFT that gives rise to the myocardium at the base of the pulmonary trunk [9]. Recent studies have revealed that human patients harboring a homozygous truncating mutation in HOXA1 may have cardiovascular malformations including great artery patterning defects, ventricular septal defects (VSD), bicuspid aortic valves (BAV) and Tetralogy of Fallot (TOF) [32,33]. Interestingly, a very recent study by Makki and Capecchi demonstrated that targeted deletion of the Hoxal gene leads to cardiovascular defects similar to those observed in human patients (Table 1) [34]. In addition, several NCC markers, including Hnf1b, Foxd3 and Zic1, were downregulated in *Hoxa1* mutants, suggesting a role for Hoxa1 in specification of cardiac NCCs. Targeted disruption of Hox1.5 (Hoxa3) leads to a spectrum of defects affecting the thymus, parathyroids, thyroid and submaxillary glands, facial cartilages, the heart and great vessels as well as muscular tissues of the throat [35]. Many of these defects arise from organs that are derived from the third and fourth pharyngeal arches and pouches. Cardiovascular defects observed were hypertrophy of the left

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ventricle and right atrium, hypoplasia of the right ventricle, patent ductus arteriosus, aortic valve stenosis and bicuspid pulmonary valve. In addition, more recent studies have shown that  $Hoxa3^{-/-}$  mutant mice exhibit carotid artery defects due to degeneration of the third arch artery by E10.5 (Table 1) [36]. In *Hoxa3* null mice, NCCs were able to normally migrate to the third pharyngeal arch, however decreased proliferation and delayed differentiation of NCCs was observed likely suggesting that *Hoxa3* may affect the NCC population to differentiate and instruct signals to the neighboring pharyngeal arches. Moreover, malformations of the great arteries derived from the fourth and sixth arch artery were not observed, likely reflecting potential compensation by Hoxa1 due to their similar pattern of expression in the premigratory NCCs. The lack of more severe cardiac malformations in *Hoxa1* and *Hoxa3* mutant mice also indicate that functional redundancy may be at play. Interestingly, Soshnikova *et al.* recently showed that deletion of either the *HoxA* or *HoxB* cluster did not result in early cardiac phenotype [37]. It was only when both the *HoxA* and *HoxB* clusters were deleted together that they observed an aggravated heart phenotype, where the embryo failed to undergo cardiac looping. It will also be interesting to clarify, in the near future, if there is some degree of intersection between *Hoxa1/b1/a3* and some of the known regulators of heart development like *Fgfs, Tbx1, Pbx* and *Meis*.

Although Hox proteins possess a homeodomain allowing them to bind A/T rich sequences, it soon became evident that they have poor affinity and specificity for DNA sequences, raising the question as to how specificity is achieved in vivo [29]. It is now well established that Hox proteins exert their function through interaction with other DNA-binding, which act as cofactors. To date, the best characterized Hox cofactors are the TALE homeodomain proteins, which include the Pbx and Meis proteins [38,39]. Although TALE cofactors are essential for regulating segmental identities during development, very little is known about their function in the heart. A role for Pbx1 in heart development was suggested by its expression in SHF progenitor cells, endocardial and mesenchymal cells of the endocardial cushions, in myocardial cells of the OFT and in cardiac NCC in mouse embryos [40,41]. Inactivation of *Pbx1* in mice leads to abnormal patterning of the great arteries and persistent truncus arteriosus, which results from failure in OFT septation [40,41]. Given that Pbx2 and Pbx3 are also expressed in a similar overlapping pattern in the heart, Stankunas et al. investigated the potential for genetic interactions in OFT development by decreasing gene dosage [41]. In summary, a spectrum of cardiovascular defects, including overriding aorta, BAV and VSD, was observed by removing one or more alleles of *Pbx1*, *Pbx2* and/or *Pbx3*, which resembles some of the anomalies seen with deletion of *Hoxa1*. It was further revealed that failure of cardiac OFT septation in *Pbx1* null embryos results from loss of *Pax3* expression in premigratory NCCs [40]. Together, these findings reveal important roles for the Pbx family members in embryonic heart development and possibly human CHDs.

Gene	Mutants	Phenotypes	References
Hoxa1	Hox-1.6 <sup>-/-</sup>	No reported cardiac phenotype	[42,43]
	Hoxal <sup>GFPneo/GFPneo</sup>	No reported cardiac phenotype	[44]
	Hoxa1 <sup>-/-</sup>	IAAB, ASC, RAA, BAV, VSD, TOF	[34,45]
Hoxa3	<i>Hox-1.5<sup>-/-</sup></i>	PDA, RV hypoplasia, hypertrophy of RA	
		and LV, stenosis of AV, bicuspid	[35]
		pulmonary valve	
	$U_{ova}2^{-/-}$	Degeneration of the 3 <sup>rd</sup> arch artery	[26]
	110xu3	Malformation of the carotid artery system	[30]
HoxA/HoxB	Hoxa <sup>-/-</sup> ;Hoxb <sup>-/-</sup>	Heart looping defects	[37]
Pbx1	$Pbx1^{-/-}$	Lethality by E15-E16. PTA and VSD	[41]
	<i>Pbx1</i> <sup>+/-</sup> ; <i>Pbx2</i> <sup>+/-</sup> ; <i>Pbx3</i> <sup>+/-</sup>	BAV	[41]
	<i>Pbx1</i> <sup>+/-</sup> ; <i>Pbx2</i> <sup>-/-</sup>	Overriding aorta, VSD, BAV, bicuspid	[41]
		pulmonary valve	[,,]
	$Pbx1^{+/-}; Pbx2^{-/-}; Pbx3^{+/-}$	TOF	[41]
Pbx2	$Pbx2^{-/-}$	No cardiac phenotype	[41]
Pbx3	$Pbx3^{-/-}$	No cardiac phenotype	[41]
Meis1	$Meis I^{-/-}$	Overriding aorta, VSD	[41]
	$\alpha$ -MHC-Cre;Meis $l^{ff}$	Increased postnatal cardiomyocyte	[/1]
		proliferation	[41]
Meis2	Zebrafish Meis2-MO	Heart looping defects	[46]

Table 1. Cardiac phenotypes associated with Hox, Pbx and Meis loss-of-function.

In addition, Meis proteins have been shown to act as major DNA binding partners of Pbx proteins, strongly indicating that they may intersect during OFT development. Consistent with this, genetic studies have shown that inactivation of *Meis1* phenocopies some of the cardiac defects observed in *Pbx* mutants such as overriding aorta, establishing a clear genetic interaction between these two genes [41]. While these findings show that Meisl is required for embryonic heart development, there is new evidence indicating a requirement for Meis1 function in the postnatal heart. Indeed, deletion of Meis1 in postnatal cardiomyocytes, using the  $\alpha MHC$ -Cre, leads to increased cardiomyocyte proliferation, through a mechanism involving cell cycle regulators [47]. Mahmoud et al. also observed that Meis1 overexpression limits postnatal heart regeneration after myocardial infarction, likely identifying Meis1 as a potential therapeutic target [47]. More recently, Meis1 and Meis2 transcripts were identified in a cardiac progenitor cell population expressing the transcription factors Nkx2-5, Tbx5 and Isl1, which are markers of the FHF and SHF [48]. The authors further identified specific cardiac enhancers that were enriched for *Meis* and/or Gata motifs, underlying a transcriptional network under the control of *Meis* factors. Consistent with a potential role in early cardiogenesis, Paige et al. identified Meis2 through analysis of temporal chromatin signatures in ES cells, which have been differentiated into the cardiac lineage [46]. These authors showed that knockdown of Meis2 in zebrafish leads to persistent cardiac looping defects, however no data concerning the role of Meis2 in the mouse has been reported up to now [46]. In addition, transcriptional profiling of chick and mouse endocardial cushions identified Meis2 in the endocardial cushions of the atrioventricular canal and OFT [49]. Even though these studies associate Meis2 as a potential regulator of epithelial-to-mesenchymal transformation and subsequent valve maturation, the generation of *Meis2* knockout mice will help investigate this possibility.

### 2. Conclusions

In this review, we have discussed the recent findings concerning the role of Hox, Pbx and Meis genes in mammalian heart development. The issue of functional redundancy among Pbx family members has also been well described for *Hoxa1* and *Hoxb1*, which synergize during patterning of the hindbrain, second pharyngeal arch and craniofacial development [50,51]. In addition, our work has shown that both *Hoxa1* and *Hoxb1* are expressed in the posterior SHF, raising the possibility that they could interact together during heart development. Further investigation involving tissue specific deletion in the mesoderm, endoderm, cardiac NCCs or SHF cells that express Hox, Pbx or Meis proteins will help address this question. In addition, it is likely that Pbx/Meis proteins genetically interact with Hox genes during heart development given the similar phenotypes of  $Hoxa1^{-/-}$ ,  $Pbx1^{-/-}$ and Meis  $1^{-/-}$  mutants. Thus, genetic approaches aimed at deleting one or more alleles of Hox. Pbx and Meis genes will provide valuable information regarding the intersection of these genes in OFT development. Even though we are starting to decipher the roles of Hox and TALE cofactors in heart development, little is known about the downstream target genes they activate. Makki and Capecchi performed a microarray on early somite-staged embryos and reported a list of genes, including some with a known function in the heart [45]. It will be of interest to identify a few common target genes, thereby providing insight into the potential mechanism underlying congenital heart defects. The continued study of genetically defined models with cardiac anomalies will prove useful in identifying the common pathways underlying cardiac OFT defects.

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## **Author Contributions**

B. L. and S.Z. wrote the paper. S.Z. made the artwork. N.B. and S.Z. made thoughtful suggestions in the preparation of the manuscript.

### **Conflicts of Interest**

The authors declare no conflict of interest.

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