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Hoxa1 and *Hoxb1* are required for pharyngeal arch artery development

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ABSTRACT

Hox transcription factors play critical roles during early vertebrate development. Previous studies have revealed an overlapping function of *Hoxa1* and *Hoxb1* during specification of the rhombomeres from which neural crest cells emerge. A recent study on *Hoxa1* mutant mice documented its function during cardiovascular development, however, the role of *Hoxb1* is still unclear. Here we show using single and compound *Hoxa1*;*Hoxb1* mutant embryos that reduction of *Hoxa1* gene dosage in *Hoxb1*-null genetic background is sufficient to result in abnormal pharyngeal aortic arch (PAA) development and subsequently in great artery defects. Endothelial cells in the 4th PAAs of compound mutant differentiate normally whereas vascular smooth muscle cells of the vessels are absent in the defective PAAs. The importance of *Hoxa1* and *Hoxb1*, and their interaction during specification of cardiac NCCs is demonstrated. Together, our data reveal a critical role for anterior *Hox* genes during PAA development, providing new mechanistic insights into the etiology of congenital heart defects.

1. Introduction

Cardiovascular development is a complex and ordered process that is spatially and temporally regulated. This process includes coordinated septation of the outflow tract (OFT) and patterning and remodeling of the pharyngeal arch arteries (PAAs). Hence, any perturbations in this process may result in a spectrum of cardiovascular abnormalities, as reflected by the high incidence of congenital heart diseases observed at birth (1–2% (Hoffman and Kaplan, 2002)). Malformations of the aortic arch, including interrupted aortic arch type-B (IAA-B), are among the most severe forms of CHD. The cause of these cardiovascular defects is often difficult to determine with certainty, nonetheless, studying factors that control cardiovascular development can help to better understand the etiology of these defects.

In mammals, the pharyngeal arches are transient bilateral bulges that develop in the cranial region of the embryo. Each arch has an external layer of ectoderm and an inner layer of endoderm, and in between these are mesenchymal neural crest-derived cells (NCCs) surrounding a mesodermal core. The heart is connected to the bilateral dorsal aorta by PAAs (Graham and Smith, 2001). The most cranial PAAs, which contribute to the vascularization of derivatives of the 1st and 2nd pharyngeal arches, largely regress while the caudal PAAs (3rd, 4th and 6th) are extensively remodeled to form the mature asymmetric aortic arch and great arteries. Involvement of the cardiac NCC, a subgroup of NCCs arising from the post-otic hindbrain, in the development of the PAAs and subsequent great arteries, has been well documented by neural crest ablation experiments in chick (Kirby et al., 1983; Kirby and Waldo, 1995; Porras and Brown, 2008). Cardiac NCCs originate from rhombomeres (r) 6, 7 and 8 and invade the 3rd, 4th and 6th pharyngeal arches to form the vascular smooth muscle layer of the PAA-derived great vessels (see (Kirby, 2007)). Ablation or genetic deletion of the cardiac NCCs results in vascular malformations, including defective OFT septation and abnormal patterning of the aortic arch arteries and great vessels, indicating that NCCs are crucial for normal development of the PAAs (Jain et al., 2011; Jiang et al., 2002; Kirby et al., 1983).

Among a variety of signaling molecules and transcription factors, several *Hox* proteins and co-factors have been described as important for development of the pharyngeal arch arteries. Indeed, *Hox* genes are involved in hindbrain patterning from which NCCs arise. Recently, Makki and Capecchi (2012) showed that *Hoxa1* impacts great artery patterning by controlling the formation of the 4th PAA (Makki and Capecchi, 2012). The use of several markers of the NCCs demonstrated that *Hoxa1* is required to specify NCCs. Interestingly, absence of another *Hox* gene, *Hoxa3*, results in anomalies of carotid arteries, deriving from the 3rd PAAs (Chisaka and Capecchi, 1991; Kameda et al., 2003). The lack of more severe cardiac malformations in *Hoxa1* and *Hoxa3* mutant mice indicates that functional redundancy may be at play. Interestingly, Soshnikova N et al. recently showed that deletion of either the *HoxA* or *HoxB* cluster did not result in a heart defect (Soshnikova et al., 2013). It

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was only when both the *HoxA* and *HoxB* clusters were deleted together that they observed an aggravated phenotype, where the heart failed to undergo looping. Furthermore, our recent works showed that paralogous *Hoxa1* and *Hoxb1* genes are expressed in cardiac progenitors contributing to arterial pole of the heart where they play a redundant role for correct OFT development (Bertrand et al., 2011; Roux et al., 2015).

Previous studies have demonstrated extensive overlapping function between *Hoxa1* and *Hoxb1* during pre-otic hindbrain specification (Gavalas et al., 1998; Rossel and Capecchi, 1999; Studer et al., 1998). We hypothesized that absence of both *Hoxa1* and *Hoxb1* genes may also have consequences on the development of PAAs, and subsequent great arteries. In the present study, we examined the possible synergy between *Hoxa1* and *Hoxb1* during PAA development through the analysis of allelic combinations. A genetic lineage tracing analysis with *Hoxa1-enhIII-Cre* and *Hoxb1*$^{IRES\text{-}Cre}$ mice showed that both genes are expressed in cardiac NCC progenitors invading the pharyngeal region. In addition, our work revealed, for the first time, that great artery defects are observed at low penetrance in embryos with loss of *Hoxb1* function. The phenotypic manifestations became more severe in the context of the additional inactivation of one *Hoxa1* allele, demonstrating that *Hoxa1* and *Hoxb1* synergize in a dosage-dependent manner during PAA development. Our analysis suggests that abnormal cardiac NCC migration is at the origin of these anomalies. We conclude that *Hoxa1* and *Hoxb1* have overlap of functions in the patterning of the rhombomeres where cardiac NCCs arise. *Hoxa1* and *Hoxb1* mutant mice thus provide a model for PAA defects often observed in CHDs.

2. Results

2.1. Hoxa1 and Hoxb1 affect great artery formation

Although *Hoxb1* is expressed in the rhombomere (r) 4 and in neural crest structures derived from the 2nd branchial arch, previous studies have not described pharyngeal arch artery (PAA) defects in *Hoxb1*-null mice (Gaufo et al., 2000; Goddard et al., 1996; Studer et al., 1996). Our recent analysis of cardiac malformations in *Hoxb1*$^{-/-}$ embryos revealed that some mutant embryos had anomalies of the OFT including misalignment and ventricular septal defects (VSD) (Roux et al., 2015). To determine if the lack of *Hoxb1* affects also great artery development we further examined *Hoxb1*$^{-/-}$ embryos at fetal stages. All wild-type (*WT*) embryos (n = 38) had normal great arteries, whereas we found a low penetrance (4%; 1 out of 28) of interruption of the aortic arch type B (IAA-B) in *Hoxb1*$^{-/-}$ embryos (Fig. 1A and B; Table 1). Since *Hoxa1* and *Hoxb1* have overlapping roles during hindbrain development, we tested for functional redundancy of the two genes in great artery development by intercrossing *Hoxa1*$^{+/-}$;*Hoxb1*$^{+/-}$ heterozygous mice (Table S1). At E17.5, all genotypes were recovered according to Mendelian ratios with the exception of *Hoxa1*$^{-/-}$;*Hoxb1*$^{-/-}$ genotype, suggesting an early lethality of double mutant embryos ($p < 0.05$ using Fisher's test, Table S1). This observation is consistent with previous studies showing a lethality of *Hoxa1*$^{-/-}$;*Hoxb1*$^{-/-}$ mouse embryos (Gavalas et al., 1998; Rossel and Capecchi, 1999). In line with a recent study, 81% of *Hoxa1*$^{-/-}$ embryos exhibited anomalies of the great arteries (Table 1) (Makki and Capecchi, 2012). These defects include IAA-B (10/27; Fig. 1C), cervical aortic arch (CAA; 7/27; Fig. 1D), aberrant retro-esophageal right subclavian artery (Ab-RSA; 4/27) and right side aortic arch (RAA; 5/27). Gross morphological observations did not reveal any difference in the great arteries of single heterozygous *Hoxa1*$^{+/-}$ or *Hoxb1*$^{+/-}$ compared to *WT* littermates. However, we found that five out of twenty-four *Hoxa1*$^{+/-}$;*Hoxb1*$^{+/-}$ embryos (21%) had great artery defects (Fig. 1E; Table 1). Interestingly, when one functional allele of *Hoxa1* was removed from a *Hoxb1*$^{-/-}$ background the number of great artery anomaly was greatly increased

Table 1
Hoxa1 and *Hoxb1* synergistically regulate cardiovascular development.

Genotype	*n*	Abnormal (n)	IAA-B	CAA	Ab-RSA	RAA
Wild-type	38	0	–	–	–	–
Hoxb1$^{+/-}$	28	0	–	–	–	–
Hoxb1$^{-/-}$	28	4% (1)	4% (1)	–	–	–
Hoxa1$^{+/-}$	34	0	–	–	–	–
Hoxa1$^{-/-}$	27	81% (22)	37% (10)	26% (7)	15% (4)	18% (5)
Hoxa1$^{+/-}$;*Hoxb1*$^{+/-}$	24	21% (5)	12% (3)	–	8% (2)	–
Hoxa1$^{+/-}$;*Hoxb1*$^{-/-}$	12	42% (5)	17% (2)	17% (2)	8% (1)	8% (1)
Hoxa1$^{-/-}$;*Hoxb1*$^{+/-}$	11	82% (9)	64% (7)	9% (1)	18% (2)	9% (1)
Hoxa1$^{-/-}$;*Hoxb1*$^{-/-}$	3	100% (3)	100% (3)	–	67% (2)	–

"Abnormal" corresponds to the total number of embryos with great artery defects. Ab-RSA, aberrant origin of the right subclavian artery including retroesophageal right subclavian artery; CAA, cervical aortic arch; IAA-B, interrupted aortic arch type B; RAA, right side aortic arch; VSD, ventricular septal defect.

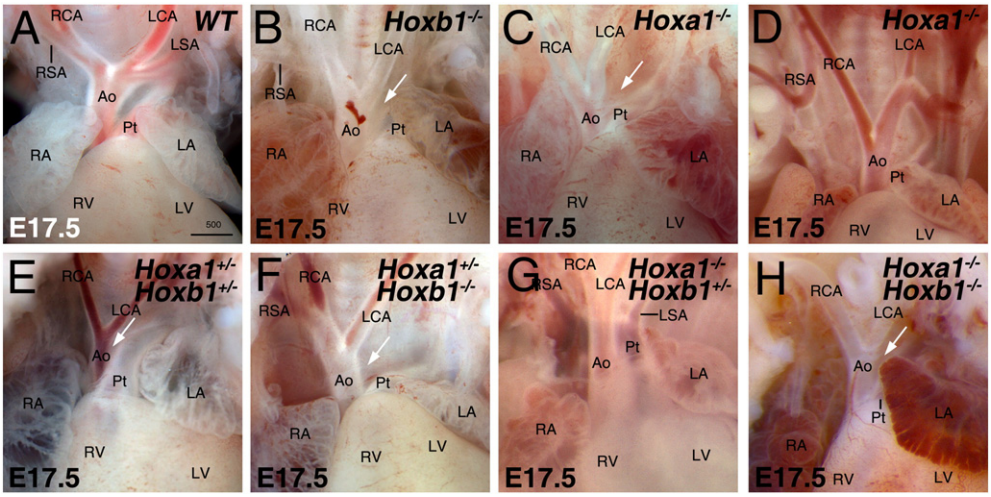

Fig. 1. Cardiovascular abnormalities in *Hoxb1*$^{-/-}$, *Hoxa1*$^{-/-}$ and compound *Hoxa1*; *Hoxb1* mutants. (A–H) Frontal views of wild-type (*WT*; A), *Hoxb1*$^{-/-}$ (B), *Hoxa1*$^{-/-}$ (C,D), *Hoxa1*$^{+/-}$;*Hoxb1*$^{+/-}$ (E), *Hoxa1*$^{+/-}$;*Hoxb1*$^{-/-}$ (F), *Hoxa1*$^{-/-}$;*Hoxb1*$^{+/-}$ (G), and *Hoxa1*$^{-/-}$;*Hoxb1*$^{-/-}$ (H) hearts at E17.5. (A) Normal pattern of the aortic arch in wild-type embryo. (B) *Hoxb1*$^{-/-}$ embryo with interrupted aortic arch type-B (IAA-B). (C, D) *Hoxa1*$^{-/-}$ embryos displaying aortic arch defects including IAA-B (C) and cervical aortic arch (D) associated with aberrant right subclavian artery (Ab-RSA). (E–H) compound *Hoxa1*;*Hoxb1* mutants showing IAA-B defects. Note the right aortic arch (RAA) in *Hoxa1*$^{-/-}$;*Hoxb1*$^{+/-}$ (G) embryo. Arrows points IAA-B defects. Ao: aorta; LCA: left carotid artery; LSA: left subclavian artery; LV: left ventricle; Pt: pulmonary trunk; RCA: right carotid artery; RSA: right subclavian artery; RV: right ventricle. Scale bar: 500 μm.