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Hand2 function in second heart field progenitors is essential for cardiogenesis

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ABSTRACT

Cardiogenesis involves the contributions of multiple progenitor pools, including mesoderm-derived cardiac progenitors known as the first and second heart fields. Disruption of genetic pathways regulating individual subsets of cardiac progenitors likely underlies many forms of human cardiac malformations. *Hand2* is a member of the basic helix loop helix (bHLH) family of transcription factors and is expressed in numerous cell lineages that contribute to the developing heart. However, the early embryonic lethality of *Hand2*-null mice has precluded lineage-specific study of its function in myocardial progenitors. Here, we generated and used a floxed allele of *Hand2* to ablate its expression in specific cardiac cell populations at defined developmental points. We found that *Hand2* expression within the mesoderm-derived second heart field progenitors was required for their survival and deletion in this domain recapitulated the complete *Hand2*-null phenotype. Loss of *Hand2* at later stages of development and in restricted domains of the second heart field revealed a spectrum of cardiac anomalies resembling forms of human congenital heart disease. Molecular analyses of *Hand2* mutant cells revealed several genes by which *Hand2* may influence expansion of the cardiac progenitors. These findings demonstrate that *Hand2* is essential for survival of second heart field progenitors and that the graded loss of *Hand2* function in this cardiac progenitor pool can cause a spectrum of congenital heart malformation.

Introduction

Congenital heart defects (CHDs) represent the most common form of human birth defects and occur in nearly 1% of live births (Hoffman and Kaplan, 2002). The recognition that individual pools of cardiac progenitors contribute to specific regions of the heart suggests that some CHDs may be due to disruption of genetic pathways that control migration, survival, expansion or differentiation of distinct populations of cells that contribute to the heart. Because of the dynamic nature of early embryonic development, it is also likely that the developmental requirement for critical genes within progenitors occurs during specific developmental windows.

Cell lineage analyses have demonstrated that the heart develops from multiple sources of cells (reviewed in Buckingham et al., 2005; Olson, 2006; Srivastava, 2006). Two progenitor cell populations, the first heart field (FHF) and second heart field (SHF) are derived from the lateral plate and splanchnic mesoderm, respectively. The third lineage is derived from cardiac neural crest (CNC) cells. In mice, the FHF forms the crescent shaped heart primordium at embryonic day (E) 7.5. At E8.0, these cells fuse at the ventral midline to form the primary heart tube and later contribute to most of the left ventricle (Buckingham et al., 2005). Meanwhile, the SHF cells, initially medial and caudal to the FHF, migrate through the pharyngeal mesoderm into the heart tube from both the anterior and posterior poles as the heart tube breaks symmetry and bends to the right. Molecularly distinct subsets of the SHF cells contribute to the outflow tract myocardium, right ventricle and atria. By E10.5, CNC cells migrate from their birthplace along the dorsal aspect of the neural folds into the outflow tract to ultimately septate the outflow into two distinct vessels, where they also differentiate into vascular smooth muscle cells.

Hand2, also known as *dHAND*, is a member of the basic helix-loop-helix (bHLH) family of transcription factors. *Hand2* is expressed in the heart, limb bud, and numerous neural crest derivatives during embryogenesis (Srivastava et al., 1995; Srivastava et al., 1997). In the heart, *Hand2* is expressed throughout the entire primary heart

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tube during early embryonic stages, with dominant expression in the right ventricle and outflow tract as the heart tube loops. *Hand2*-null (*Hand2*$^{-/-}$) mice show severe hypoplasia of the right ventricle and growth retardation from E9.5, with death by E10.5 (Srivastava et al., 1997; Yamagishi et al., 2001).

The severe phenotype and early embryonic lethality of *Hand2*$^{-/-}$ mice have precluded the determination of the precise roles of *Hand2* within the individual cell types where it is expressed. The tissue-specific deletion of *Hand2* in neural crest cells and in the limb bud has revealed essential roles in each tissue (Galli et al., 2010; Morikawa and Cserjesi, 2008). To determine the function of *Hand2* in specific subsets of myocardial progenitors that contribute to the heart as well as the cellular mechanisms underlying the severe hypoplasia of the right ventricle in *Hand2*$^{-/-}$ mice, we established and examined conditional knockout alleles of *Hand2* in specific SHF domains.

Materials and methods

Gene targeting and genotyping

To generate a conditional allele of *Hand2, loxP* sites were placed flanking the two introns of *Hand2*. For selection purposes, a neomycin cassette, flanked by two *frt* sites, was also placed upstream of the first exon. Homologous recombination and deletion of the Neo cassette by Flip-*frt* recombination created the *Hand2*^{loxp} allele (Fig. 1A). Genotyping was accomplished by digesting DNA with BamHI and ClaI and Southern analysis with a 5′ ^{32}P-radiolabeled probe as described (Srivastava et al., 1997). This process produced a 4.5-kb band representing the *Hand2*^{loxp} allele, a 5.9-kb band representing the null allele, and a 7.5-kb band representing the wild type (Fig. 1B). *Cre* driver (male) and *Hand2*$^{+/-}$ (female) mice were mated to yield *Cre:Hand2*$^{+/-}$ mice (male), which were mated with *Hand2*$^{loxp/loxp}$ mice to generate *Cre:Hand2*$^{loxp/-}$ conditional knockout mice.

Generating conditional knockout mice

Hand2^{loxp} mice were mated with four different *Cre* driver lines: *Tbx1Cre* (Maeda et al., 2006), *Mef2cCre* (Dodou et al., 2004), and *Islet1Cre* (Cai et al., 2003), which excise the floxed gene in distinct second heart field domains; and *Nkx2.5Cre* (McFadden et al., 2005), which is active in both the right and left ventricles. Each *Cre* line was crossed with *Hand2*$^{+/-}$ mice to obtain *Cre:Hand2*$^{+/-}$ males. These mice were crossed with *Hand2*$^{loxp/loxp}$, *Hand2*$^{loxp/+}$, *and Hand2*$^{loxp/+}$; *ROSA*^{LacZ} females. All mouse lines were of mixed C57BL6/129SVEJ background. We collected the resulting embryos between E8.5 and E18.5. A summary of cardiac cell types affected by each Cre line as previously published using reporter lines is provided below:

E9.5		Islet1Cre	Mef2cCre	Nkx2.5Cre	Tbx1Cre
Outflow tract	Myocardium	++	++	−	++
	Endocardium	+	++	−	+
Right ventricle	Myocardium	++	++	++	Partial
	Endocardium	+	++	+	Partial
Left ventricle	Myocardium	Partial	Partial	++	−
	Endocardium	−	−	+	−
Atrium	Right	Partial	−	−	−
	Left	−	−	−	−

Histology

The embryos from timed matings were harvested and fixed overnight in 4% paraformaldehyde/PBS. After fixation, embryos were rinsed in PBS, dehydrated overnight in 70% ethanol, and embedded in paraffin wax. Histological sections were cut and stained with hematoxylin and eosin or used for other analyses, such as TUNEL and cell-proliferation assays.

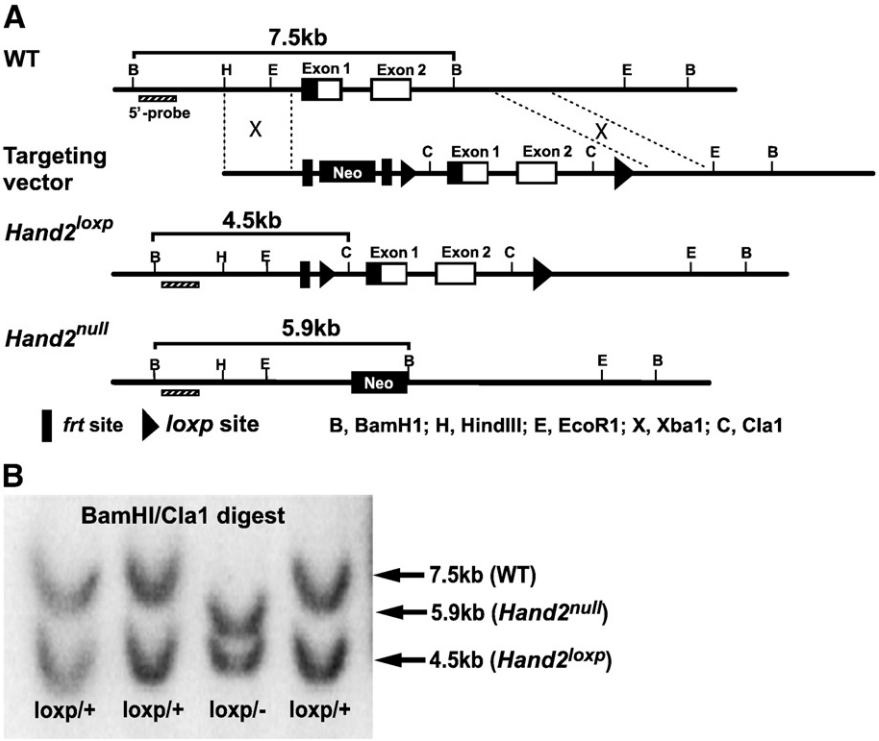

Fig. 1. Strategy for tissue-specific inactivation of *Hand2* using *Cre-loxp* system. A) Targeting strategy for generating the conditional allele of *Hand2*. In the targeting vector, a *Neo* resistance gene cassette was flanked by two *frt* sites, and two loxp sites were inserted surrounding the entire *Hand2* gene. After targeting, F1 mice were crossed with *flp* transgenic mice to remove the *Neo* cassette, resulting in the *Hand2*^{loxp} allele. We also used the previously published *Hand2*-null allele for this study. B) Genotyping was performed by Southern analysis with *BamHI* and *Cla1* digested genomic DNA. B, *BamHI*; H, *HindIII*; E, *EcoRI*; X, *XbaI*; C, *ClaI*. WT, wild type.