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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.

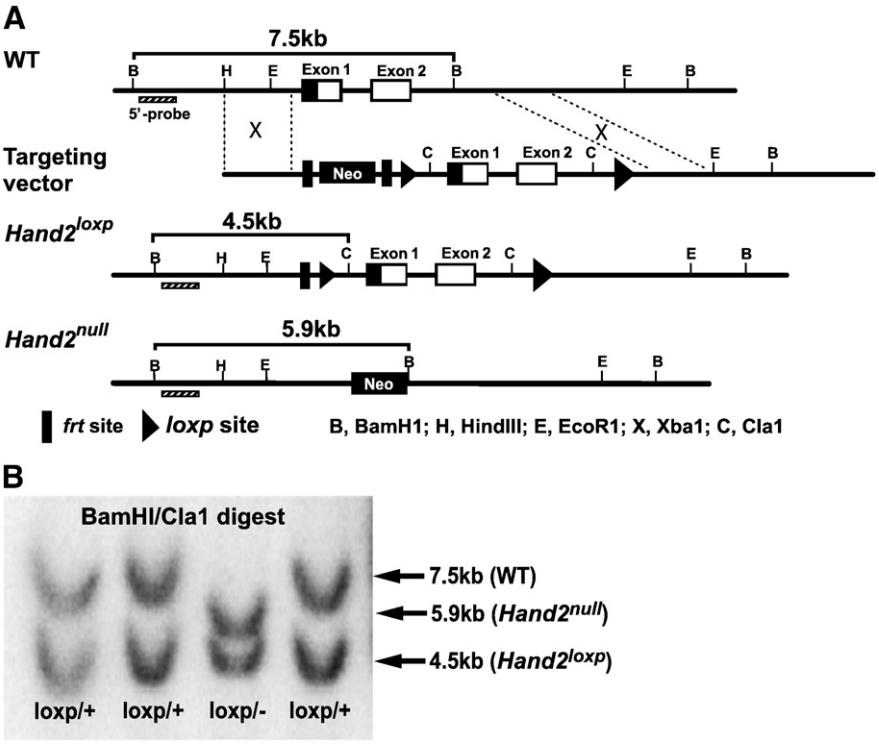


Fig. 1. Strategy for tissue-specific inactivation of *Hand2* using *Cre-loxp* system. A) Targeting strategy for generating the conditional allele of *Hand2*. In the targeting vector, a *Neo* resistance gene cassette was flanked by two *frt* sites, and two loxp sites were inserted surrounding the entire *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.

LacZ staining of embryos

The embryos from timed matings were harvested and prefixed for 2 h in 4% paraformaldehyde, 0.25% glutaraldehyde/PBS at 4 °C. Beta-galactosidase staining was performed as described (Yamagishi et al., 2003).

TUNEL assay and cell-proliferation assay

TUNEL staining and proliferation assays were performed on transverse sections from E9.0 embryos embedded in paraffin wax. The Cell Death Detection Kit (Roche) was used for TUNEL assays. Proliferation assays used primary anti-phosphohistone H3 antibody (rabbit, diluted 1:200 in 1% BSA/PBS), biotin-labeled goat anti-rabbit IgG, and FITC-strept-avidin. A statistical analysis was performed using the Student's *t*-test. A P value of 0.05 or less was considered significant.

Messenger RNA expression array analysis

E9.0 mouse hearts from wild-type, *Hand2*$^{-/-}$, or *Nkx2.5Cre:Hand2*$^{loxp/-}$ mice were harvested using Trizol reagent (Invitrogen) for total RNA isolation. The total RNA (50 ng) was labeled and hybridized to a mouse mRNA expression microarray (Affymetrix) for analysis. Gene expression values were obtained from Affymetrix CEL files with the GC-RMA package from Bioconductor.

Quantitative RT-PCR

Total RNA was isolated from E9.0 hearts with Trizol reagent (Invitrogen) and 20 ng was reverse transcribed using the Multi Reverse Transcriptase by Random Primer Labeling method (Roche). Quantitative RT-PCR (qRT-PCR) was performed using an ABI7900 with Taqman primer sets for selected cardiac developmental genes, and results were normalized to the expression of *Gapdh*. Statistical analysis was performed using the Student's *t*-test. The results shown are representative of more than three independent experiments.

Results

Generation of Hand2 floxed allele

To determine the function of *Hand2* in specific domains, we generated a conditional knockout allele of *Hand2* (Fig. 1A). Homologous recombination in mouse embryonic stem (ES) cells resulted in ES cells containing *loxp* sites flanking the *Hand2* gene and *frt* sites surrounding the *Neomycin* (*Neo*) resistance cassette 5′ of the first exon. Successful site-specific heterozygous recombination was confirmed by Southern analysis (Fig. 1B). Two distinct targeted ES cell lines were injected into blastocysts and the resulting high percentage chimeras were bred for the germline transmission of the targeted allele. Heterozygous mice were intercrossed with mice that ubiquitously expressed *Flp*-recombinase to remove the *Neo* gene (*Hand2*$^{loxp/+}$). Like *Hand2*$^{+/-}$ mice, *Hand2*$^{loxp/+}$ mice were phenotypically normal and fertile. The intercross of *Hand2*$^{loxp/+}$ mice generated *Hand2*$^{loxp/loxp}$ mice, and mice with this genotype approximated Mendelian inheritance at birth, survived to adulthood, and bred normally. The deletion of *Hand2* from the *Hand2*$^{loxp/+}$ or *Hand2*$^{loxp/loxp}$ conditional alleles depends on the expression and activity of Cre-recombinase. Several domain-specific *Cre* driver mice that express Cre-recombinase in domains of interest were mated with *Hand2*$^{+/-}$ mice and further crossed with *Hand2*$^{loxp/+}$ or *Hand2*$^{loxp/loxp}$ mice to obtain mouse embryos with domain-specific ablation of *Hand2* gene function.

Hand2 function is required in early SHF cardiac progenitor cells

We ablated *Hand2* with the *Nkx2.5*-enhancer-driven *Cre* transgenic mouse that results in Cre-recombinase activity in the right and left ventricular chambers after E8.5, but not in the earlier cardiac progenitors prior to their differentiation into cardiac cells (McFadden et al., 2005). *Nkx2.5Cre:Hand2*$^{loxp/-}$ mice formed both right and left ventricles (Fig. 2 B,F), although they were slightly smaller than those of the wild-type embryos at E9.5 (Fig. 2 A,E) and the mutant mice died by E12.5. Thus, the deletion of *Hand2* after initial specification of cardiac progenitors appeared to affect the expansion of cardiomyocytes in the embryonic heart, but to a much lesser degree than observed in the complete *Hand2*-null state.

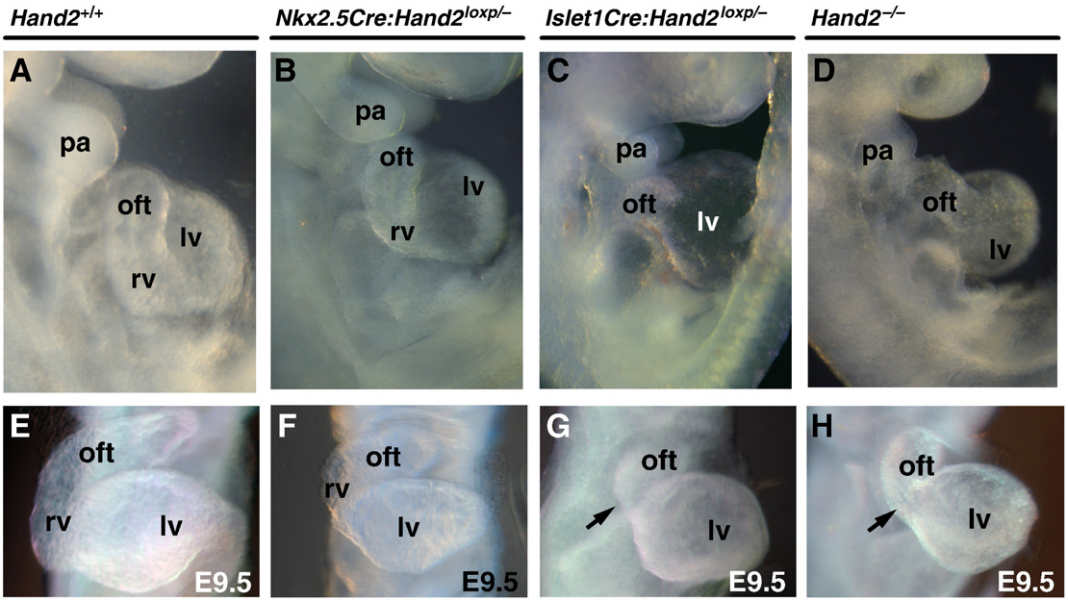


Fig. 2. *Hand2* deletion in the SHF mimics cardiac defect of the *Hand2*$^{-/-}$ germline mutant. Right lateral views (A–D) and frontal views (E–H) of the heart of embryos at E9.5. In *Nkx2.5Cre:Hand2*$^{loxp/-}$ embryos, the right ventricular (rv) segment was formed, although it was slightly smaller than that of wild type. *Islet1Cre:Hand2*$^{loxp/-}$ embryos demonstrated severe hypoplasia of the rv similar to *Hand2*$^{-/-}$ hearts (arrows in G and H). pa, pharyngeal arch; oft, outflow tract; lv, left ventricle.

Hand2 expression is high in the early cardiac progenitors of the FHF and SHF and begins to decline by E9.5 (Srivastava et al., 1995, 1997), suggesting that it might have earlier functions in cardiac progenitor cells. To investigate this, we used the *Islet1Cre* mouse that expresses Cre-recombinase in all of the early SHF cells, prior to the onset of sarcomeric gene expression (Cai et al., 2003). *Islet1Cre:Hand2*$^{loxp/-}$ embryonic hearts died by E10.5 with severe hypoplasia of the right ventricle and a shortened outflow tract (Fig. 2 C,G and 3 A–D), phenocopying complete *Hand2*$^{-/-}$ hearts (Fig. 2 D,H). These results suggest that *Hand2* expression and function in the early SHF progenitors are essential for their contribution to the developing heart and that the *Hand2*$^{-/-}$ cardiac defect (Srivastava et al., 1997) largely reflects its function in the SHF.

Hand2 is involved in survival of SHF progenitors of the pharyngeal mesoderm

To investigate the cause for right ventricular hypoplasia in the *Islet1Cre:Hand2*$^{loxp/-}$ embryos, we performed lineage analyses of the SHF cardiac progenitor cells by crossing this mouse into the *Rosa26*^{lacZ} background (Zambrowicz et al., 1997). In this system, the Cre-mediated recombination of the *Rosa26*^{lacZ} allele resulted in β-galactosidase (β-gal) production from the recombined, constitutively expressed *Rosa26* locus. Thus, β-gal staining (blue) marked derivatives of the SHF that once expressed *Islet1*. At E9.5, blue stain was detected in the pharyngeal region and outflow tract of *Islet1Cre:Hand2*$^{loxp/+}$; *Rosa26*^{LacZ} embryos, indicating that the SHF cardiac progenitor cells were migrating, as expected, from the pharyngeal mesoderm into the outflow tract (Fig. 3E). In contrast, fewer *LacZ*-positive cells were found in *Islet1Cre:Hand2*$^{loxp/-}$; *Rosa26*^{LacZ} embryos at E9.5 (Fig. 3F). The reduction was especially pronounced in the pharyngeal region of these embryos, suggesting that the decrease in progenitor cell number occurred before their migration into the outflow tract.

To determine if the decreased number of progenitor cells from the SHF resulted from abnormal cell death or decreased proliferation, we performed TUNEL and proliferation assays on the embryos collected at E9.0. More cell death was detected in the pharyngeal mesoderm of both *Islet1Cre:Hand2*$^{loxp/-}$ and *Hand2*$^{-/-}$ mutants than in the wild type embryos (Fig. 3G), while the number of TUNEL positive cells in the outflow tract was only significantly greater in the *Hand2*$^{-/-}$ embryos (Fig. 3H), likely reflecting the influence of *Hand2* in neural crest derivatives in the outflow tract as previously reported (Thomas et al., 1998). Immunohistochemical analyses using anti-Ph3 antibody revealed no significant difference in cell proliferation among the wild-type, *Islet1Cre:Hand2*$^{loxp/-}$ and *Hand2*$^{-/-}$ embryos (data not shown). These results suggest that *Hand2* is required within the SHF progenitor cells to ensure their survival and that the severe right ventricular hypoplasia that occurs from the loss of *Hand2* is a result of enhanced apoptosis of the SHF cells within the pharyngeal mesoderm before they contribute to the heart.

Loss of Hand2 in subsets of SHF progenitors causes a spectrum of right heart lesions

The discovery of distinct subpopulations of SHF progenitors raises the possibility that some forms of CHD involving the outflow tract or right ventricle are caused by the loss of some or all SHF cells during cardiogenesis. Having demonstrated that *Hand2* was essential for the survival of SHF progenitors, we investigated whether the loss of *Hand2* in smaller subsets of SHF could result in cardiac anomalies more similar to CHDs in humans. Transgenic mice with Cre-recombinase under the control of the SHF-specific enhancer of *Mef2c* (*Mef2cCre*) express Cre-recombinase in a distinct subset of the SHF-derived cells that is narrower than that of the *Islet1Cre* driver mice, encompassing the outflow tract and right ventricle, including the ventricular septum (Dodou et al., 2004). The *Mef2cCre:Hand2*$^{loxp/-}$ embryos had no gross growth retardation until E12.5, but died around E13.5. By morphological analyses, the right ventricle was smaller in the *Mef2cCre:Hand2*$^{loxp/-}$ mutant embryos than in the wild type

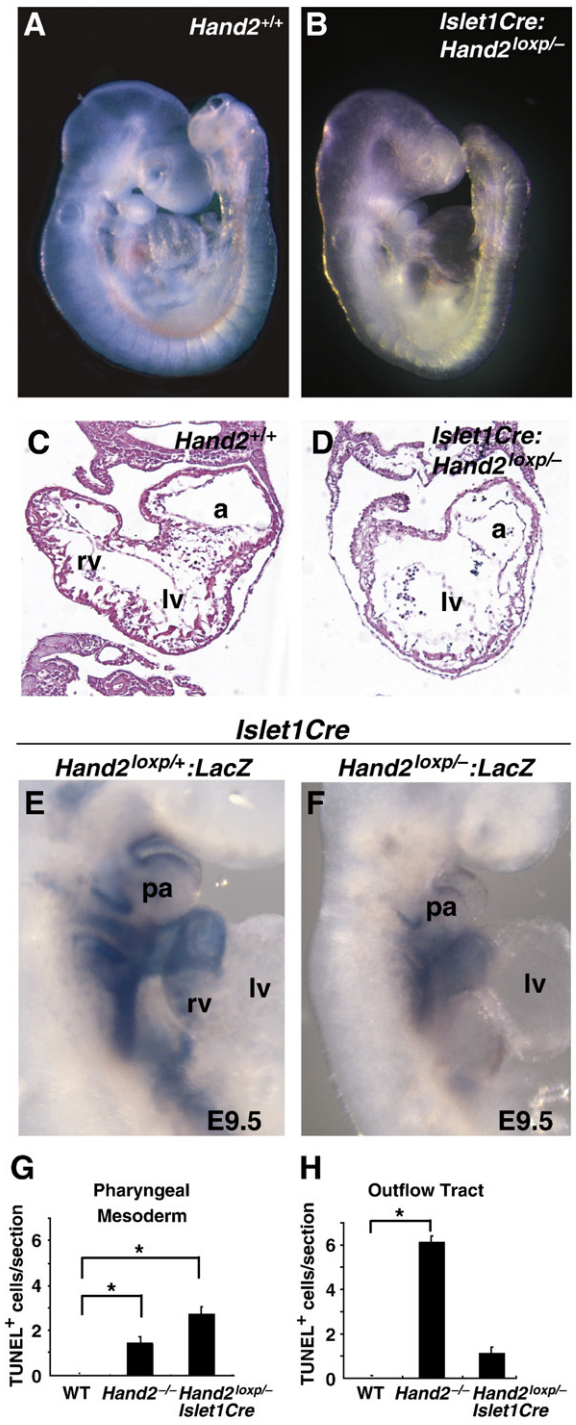


Fig. 3. *Hand2* deletion in the SHF results in reduced number of cardiac progenitor cells in the pharyngeal region. Right lateral views of wild-type (A) or *Islet1Cre:Hand2*$^{loxp/-}$ (B) embryos at E9.5. Growth retardation of mutant embryos was noted. Transverse section from wild-type (C) or *Islet1Cre:Hand2*$^{loxp/-}$ (D) embryos at E9.5 demonstrated severe hypoplasia of right ventricle (rv) and a thin walled left ventricle (lv) in mutants, similar to *Hand2*-nulls. Compared with *Islet1Cre:Hand2*$^{loxp/+}$; *Rosa26*^{LacZ} embryo (E), LacZ staining was reduced in *Islet1Cre:Hand2*$^{loxp/-}$; *Rosa26*^{LacZ} (F), indicating a reduced number of cardiac progenitor cells in the SHF of this mutant. Results of TUNEL assay on wild type, *Hand2*$^{-/-}$, and *Islet1Cre:Hand2*$^{loxp/-}$ E9.0 embryos in the pharyngeal mesoderm (G) and the outflow tract (H) are shown. Excessive cell death was observed in the pharyngeal mesoderm of *Islet1Cre:Hand2*$^{loxp/-}$ and *Hand2*$^{-/-}$ embryos, and in the outflow tract of *Hand2*$^{-/-}$ embryos. pa, pharyngeal arch; a, atrium. *p = 0.05.

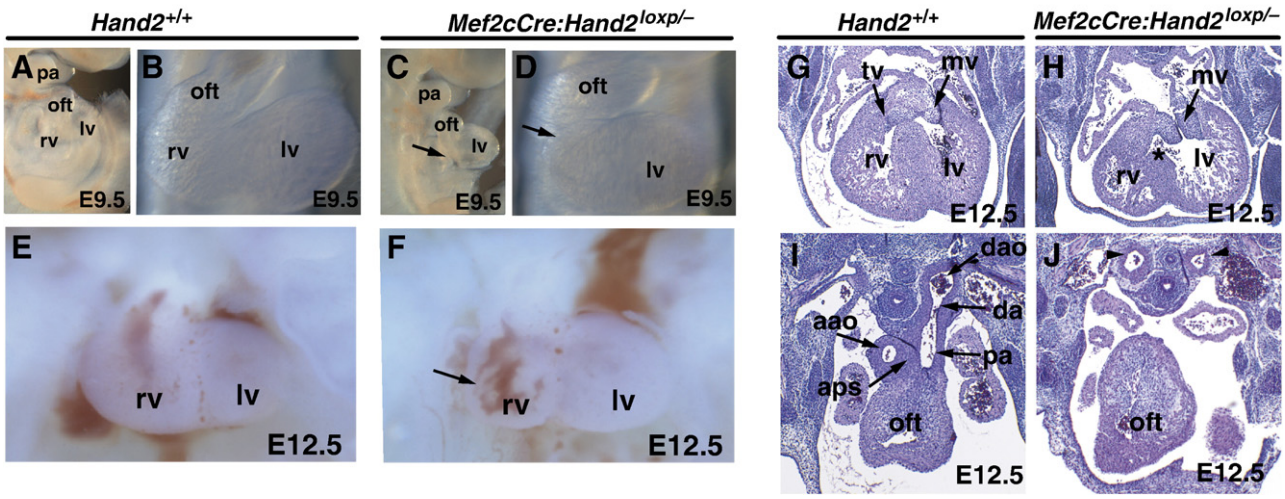


Fig. 4. Hypoplastic right ventricle with tricuspid atresia in *Mef2cCre:Hand2* conditional knockout mice. Wild-type (A, B) and *Mef2cCre:Hand2*$^{loxp/-}$ hearts (C, D) at E9.5 are shown in right lateral (A, C) and frontal views (B, D). Wild-type (E) and *Mef2cCre:Hand2*$^{loxp/-}$ hearts (F) at E12.5 are shown in frontal views. A small and thin right ventricle (rv) was observed in *Mef2cCre:Hand2*$^{loxp/-}$ (arrows in C, D, F). Transverse sections of ventricular level (G, H) or outflow tract (oft) level (I, J) in hearts of wild-type (G, I) or *Mef2cCre:Hand2*$^{loxp/-}$ (H, J) embryos. *Mef2cCre:Hand2*$^{loxp/-}$ hearts showed a small rv, interventricular septal defect (asterisk in H) and no evidence of a tricuspid valve (tv). While wild type demonstrated appropriate asymmetric remodeling for primitive aortic arches into ascending aorta (aao), main pulmonary artery (pa), ductus arteriosus (da), and descending aortic arch (dao) (I), *Mef2cCre:Hand2*$^{loxp/-}$ mutants displayed bilateral descending aortas (arrowheads in J) with no evidence of outflow septation. lv, left ventricle; mv, mitral valve; aps, aortico pulmonary septum.

embryos at E9.5 (Fig. 4 A–D), but larger than the *Hand2*$^{-/-}$ or *Islet1Cre:Hand2*$^{loxp/-}$ right ventricles. Analyses of these mutants at E12.5 revealed a hypoplastic right ventricle with relatively thin myocardium (Fig. 4 E–H). The *Mef2cCre:Hand2*$^{loxp/-}$ mutant embryos lacked the anlage of a tricuspid valve between the right atrium and ventricle in any level of section, and displayed ventricular septal defects (Fig. 4H). This anatomy is reminiscent of a type of human CHD known as tricuspid atresia. At this stage, the distal outflow tract was divided into two vessels in the wild type (Fig. 4I), but an immature truncus arteriosus was noted in *Mef2cCre:Hand2*$^{loxp/-}$ mutants (Fig. 4J), probably as a result of the delayed remodeling of the outflow tract and aortic arches.

We previously showed that the transcription factor *Tbx1* is expressed in cells whose descendants contribute mainly to the distal right ventricular outflow tract and pulmonary artery (Yamagishi et al., 2003; Maeda et al., 2006). We used an enhancer of *Tbx1* driving *Cre-recombinase* (*Tbx1-Cre*) to delete *Hand2* in this narrower subset of the SHF-derived cells (Maeda et al., 2006). The analyses of *Tbx1Cre:Hand2*$^{loxp/-}$ mutants at E10.5 revealed a shortened outflow tract, while the right ventricle appeared to be developing normally (Fig. 5 A–D). By E13.5, the *Tbx1Cre:Hand2*$^{loxp/-}$ embryos had smaller right ventricles and outflow tracts than the wild-type mice (Fig. 5 E,F) and died by E15.5.

We used the *Rosa26*^{LacZ} mouse to lineage trace the consequence of *Hand2* deletion in *Tbx1*-expressing cells. *LacZ* staining of E12.5 *Tbx1Cre:Hand2*$^{loxp/-}$; *Rosa26*^{LacZ} mutant embryos highlighted the narrowing of the outflow tract and hypoplasia of the outlet, or subpulmonary conus, of the right ventricle compared to *Tbx1Cre:Hand2*$^{loxp/+}$; *Rosa*^{LacZ} littermates (Fig. 5 G–J). The transverse sections of embryos at E14.5 further demonstrated that the right ventricular cavity of *Tbx1Cre:Hand2*$^{loxp/-}$ mutants was smaller than the wild type, although the right ventricular muscle wall thickness was comparable (Fig. 5 K,L). The decreased chamber volume was likely secondary to hypoplasia of the infundibular components, as the pulmonary valve appeared normal (Fig. 5 M,N). Notably, the relationships of the great vessels to the ventricular chambers and the ventricular septum were normal in all of the mutant embryos. Hypoplasia of the infundibular components (outlet portion) of the right ventricle is often observed in many forms of human CHD and may reflect loss of the distal SHF progenitors.

Analyses of Hand2-dependent cardiac gene expression

To elucidate the gene expression alterations that may underlie the severe right ventricular hypoplasia in *Hand2*$^{-/-}$ mice and *Islet1Cre:Hand2*$^{loxp/-}$ mice, we performed an mRNA expression array analysis that compared the E9.0 wild-type, *Hand2*$^{-/-}$, and *Nkx2.5Cre:Hand2*$^{loxp/-}$ embryonic hearts. Since severe right ventricular hypoplasia was not demonstrated in the *Nkx2.5Cre:Hand2*$^{loxp/-}$ animals, we hypothesized that genes dysregulated in the *Hand2*$^{-/-}$ hearts, but not in the *Nkx2.5Cre:Hand2*$^{loxp/-}$ hearts, may be implicated in the defects specifically resulting from ablation of *Hand2* in the SHF.

We selected several key regulators of cardiac development that appeared to be dysregulated by microarray (Fig. 6A) and performed qRT-PCR analyses to validate the array results. Interestingly, expression levels of *Gata4*, *Has2* and *Bmp5* were significantly downregulated in the *Hand2*$^{-/-}$ hearts, but not in the *Nkx2.5Cre:Hand2*$^{loxp/-}$ hearts, by array (Fig. 6B) and qRT-PCR (Fig. 6 C–E). Decreased levels of *Has2* in the *Hand2*$^{-/-}$ hearts may reflect the smaller right ventricle since *Has2* is more enriched in this domain; however, the downregulation of *Gata4* and *Bmp5*, among other dysregulated genes, may contribute to the severe right ventricle hypoplasia observed in *Hand2*$^{-/-}$ or *Islet1Cre:Hand2*$^{loxp/-}$ mice. The downregulation of *Hand2* was also confirmed in both *Hand2*$^{-/-}$ and *Nkx2.5Cre:Hand2*$^{loxp/-}$ hearts by qRT-PCR (Fig. 6F) and other genes dysregulated in both settings such as Klf4 and Klf7 may contribute to the expansion defect.

Discussion

We sought to dissect the roles of *Hand2* in the various cardiac progenitor cells where it is normally expressed and to gain an understanding of the process by which *Hand2* controls heart development. We created a floxed *Hand2* allele, which we used to delete *Hand2* in specific domains. Through the analyses of these mutant mice, we found that *Hand2* was required in the SHF progenitors for their early survival. Temporal- or spatially-restricted ablation of *Hand2* function in the SHF revealed its necessity for the normal development of the outflow tract, tricuspid valve, and ventricular septum. Interestingly, the abnormalities seen in mice with conditional ablation of *Hand2* in discrete sub-domains shared some resemblance to several human congenital heart malformations. The gene expression analyses of these

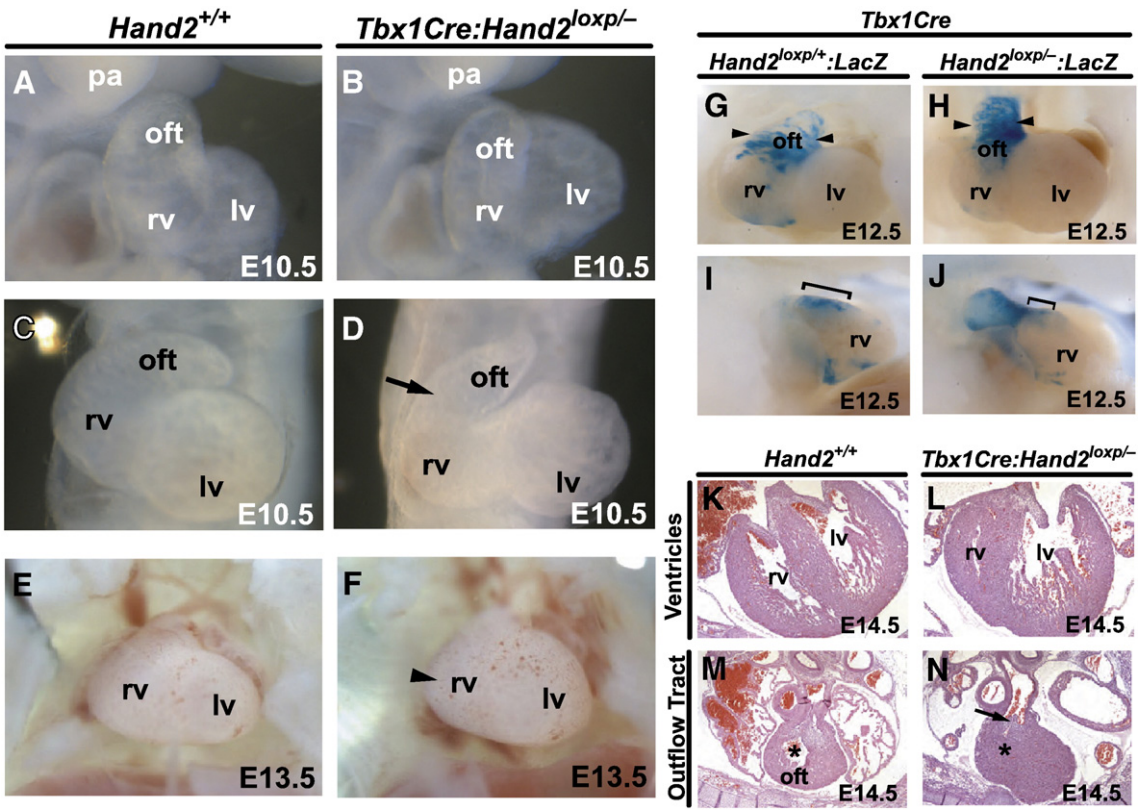


Fig. 5. Subpulmonary conus defect in *Tbx1Cre:Hand2* conditional knockout mice. Right lateral views (A, B) and frontal views (C, D) of the heart at E10.5 and frontal views at E13.5 (E, F) of wild type (A, C, E) and *Tbx1Cre:Hand2*$^{loxp/-}$ embryos (B, D, F). The outflow tract (oft) was shorter in the *Tbx1Cre:Hand2*$^{loxp/-}$ (arrow in D) than in the wild type. Note the small right ventricle (rv) in *Tbx1Cre:Hand2*$^{loxp/-}$ at E13.5 (arrowhead in F). Frontal (G, H) and right lateral (I, J) views of *Tbx1Cre:Hand2*$^{loxp/+}$*: Rosa*^{LacZ} (G, I) and *Tbx1Cre:Hand2*$^{loxp/-}$*: Rosa*^{LacZ} (H, J) embryos with *lacZ* staining at E12.5. Narrowing (between arrowheads in G, H) and shortening of the oft (brackets in I, J) could be seen in *Tbx1Cre:Hand2*$^{loxp/-}$*: Rosa*^{LacZ}. Transverse section at ventricular level (K, L) or outflow tract level (M, N) of wild type (K, M) or mutant (L, N) at E14.5. Small rv in *Tbx1Cre:Hand2*$^{loxp/-}$ compared to wild type (K). Wild type embryos had a right ventricular oft lumen (asterisk in M), whereas *Tbx1Cre:Hand2*$^{loxp/-}$ had no evidence of the lumen (asterisk in N) below the pulmonary valve (arrow in N) at any level of section, suggesting the absence of infundibular components. pa, pharyngeal arch; lv, left ventricle.

mutant mice revealed potential effectors of *Hand2* in right ventricular and general myocardial development.

Hand2 is essential in the SHF for right ventricular development

In this study, we found that the loss of *Hand2* in the broadest SHF domain phenocopies the ventricular defect observed in global *Hand2* knockout, while its ablation in a subset of SHF and FHF is not sufficient to phenocopy the *Hand2*$^{-/-}$ mutant hearts. We conclude that the SHF is the critical region of *Hand2* expression required for proper development of the right ventricle and that this requirement is prior to the migration of the SHF cells into the heart. Intriguingly, the loss of *Hand2* in the SHF led to enhanced apoptosis of cardiac progenitor cells in the pharyngeal mesoderm before they participated in the process of cardiac development, resulting in severe hypoplasia of the right ventricle. Thus, it is likely that *Hand2* is essential for the survival of undifferentiated cardiac progenitor cells in the SHF. The deletion of *Hand2* after cardiac differentiation had begun using the ventricular-specific *Nkx2.5-Cre* transgenic mouse resulted in a later defect of ventricular expansion, similar to that observed upon the deletion of *Hand2* in the domain of the myocardial sarcomeric gene, *cardiac Troponin T* (Morikawa and Cserjesi, 2008).

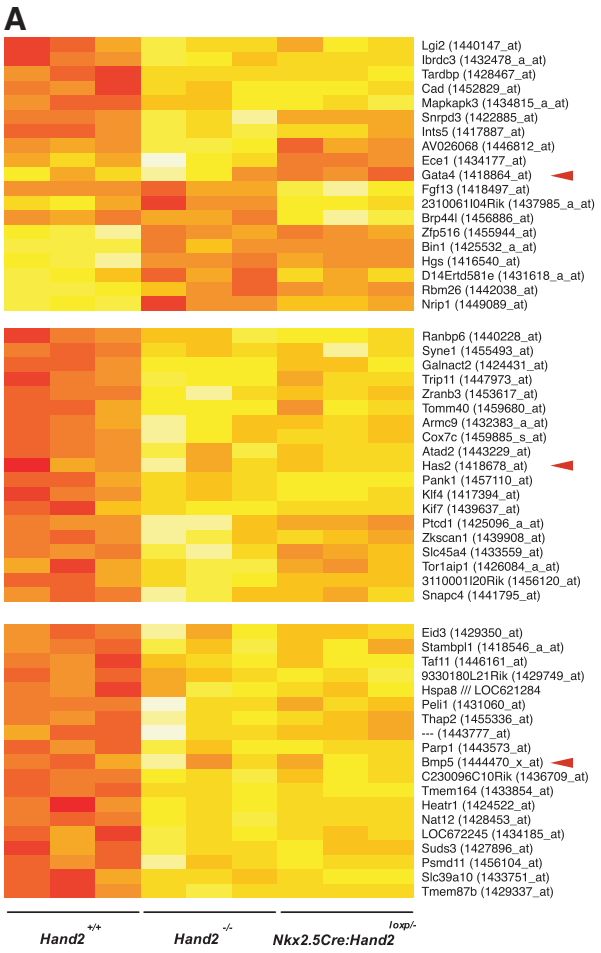
Similarity between Hand2 conditional knockout hearts and human CHDs

Deleting *Hand2* in a subset of the SHF cells resulted in various degrees of hypoplastic right heart syndrome that correlated with the breadth of the SHF cell population that was targeted. For example, the *Tbx1Cre:Hand2*$^{loxp/-}$ phenotype was similar to severe right ventricular outflow tract obstruction that retards right ventricular chamber growth. In contrast, *Mef2cCre:Hand2*$^{loxp/-}$ mice developed small, thin-walled right ventricles and tricuspid valve atresia, likely reflecting the broader deletion of *Hand2* in cells that contribute to most of the right ventricular chamber.

While homozygous deletion of *Hand2* has not been described in humans, several reports indicate that the *Hand2* heterozygous deletion is part of 4q syndrome, which involves developmental craniofacial, musculoskeletal, and cardiac defects. 4q33, the *Hand2* locus (Natarajan et al., 2001), has been identified as the critical region of 4q syndrome (Towns et al., 1979; Yu et al., 1981), and CHDs were diagnosed in half of the 4q syndrome patients. The defects included pulmonary valve stenosis or atresia, patent ductus arteriosus, tetralogy of Fallot, tricuspid valve atresia, and coarctation of the aorta. (Keeling et al., 2001; Strahle and Middlemiss, 2007). Some of these may reflect *Hand2*'s function in the neural crest, as neural crest-specific ablation of *Hand2* causes similar loss of neural crest derivatives in the heart (Morikawa and Cserjesi, 2008) and the deletion of the pharyngeal arch-specific *Hand2* enhancer causes craniofacial defects (Yanagisawa et al., 2003). The overlapping phenotypes of *Hand2* SHF ablation in mice and 4q syndrome in humans suggests a potential role for *Hand2* in human CHDs, and points to the relevance of abnormal SHF progenitor cell development to human heart disease.

Putative downstream targets of Hand2 implicated in ventricular development

In an effort to examine gene expression defects in the SHF of *Hand2*$^{-/-}$ mice, we found that the expression of the zinc finger



B

Gene	Mouse Genotype		
	$Hand2^{+/+}$	$Hand2^{-/-}$	$Nkx2.5Cre:Hand2^{loxp/-}$
Gata4	1.000	0.398	0.791
Has2	1.000	0.315	0.787
Bmp5	1.000	0.208	0.849

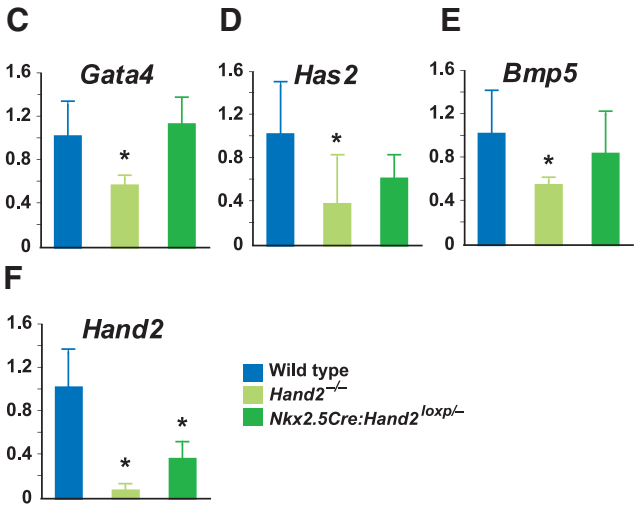


Fig. 6. mRNA microarray analysis of *Hand2* mutant hearts. (A) Microarray studies comparing gene expression profiles in wild-type versus $Hand2^{-/-}$ and $Nkx2.5Cre:Hand2^{loxp/-}$ mouse embryonic hearts. Heat map represents a portion of the genes downregulated in $Hand2^{-/-}$ and $Nkx2.5Cre:Hand2^{loxp/-}$, highlighting Gata4, Has2, and Bmp5 (arrowheads). Red-yellow gradient shows decrease in heat map value. (B) Results from mRNA expression microarray using wild-type, $Hand2^{-/-}$ and $Nkx2.5Cre:Hand2^{loxp/-}$ mouse embryonic hearts of selected cardiac development genes. Each number is a ratio of expression level compared with wild-type set to 1.0. (C–F) qPCR analyses were performed to validate the results of the microarray analysis for *Gata4* (C), *Has2* (D), *Bmp5* (E), and *Hand2* (F). Asterisks denote significant changes in gene expression ($p<.05$) compared with wild type.

transcription factor, *Gata4*, was reduced in the $Hand2^{-/-}$ hearts, but not in $Nkx2.5Cre:Hand2^{loxp/-}$. Interestingly, *Gata4* expression was also unchanged in mice containing a specific mutation in the *Hand2* DNA-binding domain, and these mice lived to a similar stage as the the $Nkx2.5Cre:Hand2^{loxp/-}$ mice (Liu et al., 2009). *Gata4* is normally expressed in the SHF and directly regulates *Hand2* during right ventricular development (McFadden et al., 2000), and *Gata4* conditional knockout mice demonstrate severe hypoplasia of the right ventricle with reduced expression of *Hand2* (Zeisberg et al., 2005). Thus, there may be a reinforcing loop between *Hand2* and *Gata4* that amplifies the expression of both genes early during SHF and subsequent right ventricular development.

In addition to *Gata4*, the expression of *Has2* and *Bmp5* was reduced in the $Hand2^{-/-}$ hearts compared to the wild type and $Nkx2.5Cre:Hand2^{loxp/-}$. *Has2* encodes hyaluronan synthase-2, which is essential for hyaluronic acid secretion into the extracellular matrix between the early endocardium and myocardium, known as the cardiac jelly (Camenisch et al., 2000; Mjaatvedt et al., 1998). The cardiac jelly mediates reciprocal signaling between the endocardium and myocardium that results in myocardial cell proliferation and maturation. Reduced expression of Has2 is associated with hypoplastic right ventricle (Mjaatvedt et al., 1998) and may also contribute to the abnormalities in $Hand2^{-/-}$ or $Islet1Cre:Hand2^{loxp/-}$ mice. However, it is also possible that the decrease in Has2 and other genes uniquely expressed in the right ventricle simply reflects the lack of right ventricular cells in the developing heart.

The bone morphogenic protein (BMP) family of signaling molecules mediates the simultaneous activation of Smad and Wnt signal transduction in the cardiogenic pathway, including induction of the earliest markers of cardiac differentiation, such as *Gata4*, *Nkx2.5*, and *Mef2c* (Pal and Khanna, 2006). In chick embryos, *Bmp5* is expressed in the outflow tract myocardium and may play a role in the process of the recruitment of cardiomyocytes to the outflow tract (Somi et al., 2004). Indeed, the loss of Bmp5 and Bmp7 resulted in outflow tract defects (Solloway and Robertson, 1999), suggesting that the reduced *Bmp5*-mediated signaling could contribute to the decreased numbers of SHF progenitors observed upon the reduction of *Hand2* dosage. It remains to be determined whether *Gata4*, *Has2* or *Bmp5* is directly or indirectly regulated by *Hand2*, but the regulation of Gata4 appears to be DNA binding-independent (Liu et al., 2009).

Conclusions

By establishing a conditional knockout allele of *Hand2* utilizing a variety of unique Cre-driver mouse lines, we have revealed some tissue-specific roles of the *Hand2* gene during the development of early cardiac progenitors. More importantly, the loss of *Hand2* in specific sub-domains of the SHF supports the idea that some forms of CHD might be a result of insufficient cells derived from discrete pools of progenitor cells. Our identification of genes that are specifically dysregulated when early SHF cells lack *Hand2* and are unaffected in the later knockout offers an opportunity to focus on pathways

uniquely important for the survival and expansion of cells populating the right ventricle. Further investigation of the function of these factors may contribute to the identification of the underlying causes of an array of CHDs affecting the human population.

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