The Hand1 and Hand2 transcription factors regulate expansion of the embryonic cardiac ventricles in a gene dosage-dependent manner

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Summary
The basic helix-loop-helix transcription factors Hand1 and Hand2 display dynamic and spatially restricted expression patterns in the developing heart. Mice that lack Hand2 die at embryonic day 10.5 from right ventricular hypoplasia and vascular defects, whereas mice that lack Hand1 die at embryonic day 8.5 from placental and extra-embryonic abnormalities that preclude analysis of its potential role in later stages of heart development. To determine the cardiac functions of Hand1, we generated mice harboring a conditional Hand1-null allele and excised the gene by cardiac-specific expression of Cre recombinase. Embryos homozygous for the cardiac Hand1 gene deletion displayed defects in the left ventricle and endocardial cushions, and exhibited dysregulated ventricular gene expression. However, these embryos survived until the perinatal period when they died from a spectrum of cardiac abnormalities. Creation of Hand1/2 double mutant mice revealed gene dose-sensitive functions of Hand transcription factors in the control of cardiac morphogenesis and ventricular gene expression. These findings demonstrate that Hand factors play pivotal and partially redundant roles in cardiac morphogenesis, cardiomyocyte differentiation and cardiac-specific transcription.

Key words: Mouse, Hand1, Hand2, Cardiac ventricles

Introduction
Cardiac malformations resulting from abnormalities in development of the embryonic heart represent the most common form of birth defects and the most prevalent cause of miscarriages in humans (Hoffman and Kaplan, 2002). There has been substantial progress in defining the morphogenic events involved in heart formation and in identifying cardiac developmental control genes (reviewed by Fishman and Chien, 1997; Harvey, 2002; McFadden and Olson, 2002; Olson and Schneider, 2003). However, there are major gaps in understanding the interconnections between cardiogenic transcription factors and their downstream effector genes that mediate cardiac myogenesis, morphogenesis and function.

Heart development begins when mesodermal cells in a region of the embryo known as the cardiac crescent become instructed to adopt a cardiac fate in response to signals from adjacent tissues (Marvin et al., 2001; Schneider and Mercola, 2001; Schultheiss et al., 1997; Tzahor and Lassar, 2001) (reviewed by Olson, 2002). Cardiac precursors proliferate and migrate to the embryonic midline to form a linear heart tube that is segmentally patterned along its anteroposterior axis into regions ultimately giving rise to the atrial and ventricular chambers. Rightward looping of the linear heart tube followed by balloon-like growth of the outer curvatures of the ventricular segments generates the right and left ventricular chambers (Christoffels et al., 2000; Moorman et al., 2000). Notably, each cardiac chamber possesses distinct physiological functions and patterns of gene expression.

Recent studies have revealed two populations of cardiac precursor cells that contribute to different parts of the heart. The primary heart field is thought to give rise to the atrial chambers and left ventricular region. A second cardiogenic region, known as the anterior or secondary heart field, lies anterior and dorsal to the linear heart tube. Cells from this region are added to the developing heart tube and give rise to the outflow tract and right ventricular region (Mjaatvedt et al., 2001; Waldo et al., 2001) (reviewed by Kelly and Buckingham,
expression of Hand1 survive until the perinatal period when they succumb to a spectrum of congenital heart defects that reflect abnormalities in ventricular growth and maturation. In addition, combination of the conditional Hand1 mutation with a Hand2 loss-of-function mutation revealed dose-sensitive effects on heart development. These results identify novel functions of Hand1 in heart development and demonstrate that Hand factors play crucial and partially redundant roles in cardiac growth, morphogenesis and gene expression.

Materials and methods

Gene targeting

A previously characterized Hand1 genomic clone (Firulli et al., 1998), was used to generate the Hand1NeomloxP targeting vector. A 2.9 kb fragment extending upstream from the Hand1 5′-UTR (long arm of homology) was amplified by high-fidelity PCR (Stratagene Pfu Turbo) and digested with XhoI. An EcoRI restriction site was engineered into the Hand1 5′UTR to facilitate genotyping by Southern blotting. This fragment was ligated into the pDelboy targeting backbone upstream of the FRT-flanked neomycin resistance cassette. A ClaI-KpnI linked fragment containing the first exon of Hand1, extending from the Hand1 5′-UTR to the intron, was amplified using high fidelity PCR and cloned into pDelboy-LA. Finally, the short arm of homology, extending from the intron to a 3′ SalI site, was PCR amplified and cloned into SalI-EcoRI digested pDelboy-LaEh upstream of the thymidine kinase negative selection cassette. Integrity of the targeting vector was confirmed by restriction mapping and DNA sequencing. PCR primer sequences are available upon request.

The completed Hand1NeomloxP targeting vector was linearized with NcoI and electroporated into SM-1 ES cells. Following positive-negative selection with G418 and FIAU, resistant colonies were screened by Southern analysis of EcoRI-digested genomic DNA using a probe (Fig. 1B) from the 3′ flanking region. Recombination of the 5′ arm was confirmed by EcoRI-KpnI double digestion of genomic DNA, and Southern blotting with the short arm of homology. Three correctly targeted clones (clones E12, C12 and C5) were expanded and injected into C57BL/6 blastocysts, and transferred into the uteri of pseudopregnant females. Chimeric males were bred onto a C57BL/6 or Black/Swiss background for germline transmission. Males from clone E12 transmitted the targeted allele through the germline, therefore mice derived from this line were used in all analyses.

Heterozygous Hand1NeomloxP mice were intercrossed with ActC:FPFP transgenic mice (Rodriguez et al., 2000) in order to remove the neomycin resistance cassette in the germline. Removal of the neomycin cassette was confirmed by Southern blotting using EcoRI-digested genomic DNA (Fig. 1B). The Hand1NeomloxP allele was bred to homozygosity, which did not affect viability or fertility of these mice, suggesting that the FRT and loxp sites in the 5′-UTR did not significantly alter expression from the targeted locus.

Generation of Nkx2.5::Cre mice

A 2.5 kb fragment containing the Nkx2.5 basal promoter and cardiac enhancer (Lien et al., 1999) was cloned upstream of the NLS-Cre expression cassette (gift of J. Herz). This vector was linearized using NotI and injected into fertilized oocytes as previously described (McFadden et al., 2000). Founder transgenic mice were genotyped by hybridization of EcoRV digested tail DNA to a Cre CDNA probe. Three transgenic lines were obtained and intercrossed with Rosa26R indicator mice (Soriano, 1999) in order to assess transgene expression and Cre-mediated recombination. Transgenic line Nkx9 exhibited the earliest and most efficient recombination, and was used in all subsequent experiments.

Several classes of transcription factors have been implicated in cardiac morphogenesis and gene regulation (reviewed by Bruneau, 2002; Firulli and Thallalayath, 2002). Hand1 and Hand2 (also called eHAND/Thing-1/Hx and dHAND/Thing-2/Hed, respectively) are basic helix-loop-helix (bHLH) transcription factors that display complimentary and overlapping expression patterns in the developing heart (Cross et al., 1995; Hollenberg et al., 1995; Cserjesi et al., 1995; Srivastava et al., 1995). In mice, Hand2 is expressed throughout the linear heart tube. Thereafter, its expression is highest in the developing right ventricle (RV), with lower levels of expression in the atrial and left ventricular chambers (Thomas et al., 1998). Targeted mutation of Hand2 in mice results in lethality at embryonic day 10.5 (E10.5) from right ventricular hypoplasia and vascular malformations (Srivastava et al., 1997; Yamagishi et al., 2000). By contrast, Hand1 is expressed in segments of the linear heart tube destined to form the conotruncus and left ventricle (LV). At the onset of cardiac looping, Hand1 expression becomes localized primarily to the outer curvature of the LV and outflow tract, with lower expression along the outer curvature of the developing RV (Biben and Harvey, 1997; Thomas et al., 1998). Mice lacking Hand1 die at E8.5 from severe placental and extra-embryonic defects, reflecting a role of Hand1 in trophoblast differentiation, and complicating analysis of potential cardiac functions (Firulli et al., 1998; Riley et al., 1998). Nevertheless, tetraploid aggregation experiments with wild-type and Hand1 null embryonic stem (ES) cells have shown that mutant ES cells fail to contribute to the LV of chimeric mouse embryos. Such embryos survive until E10.5 when they exhibit abnormalities in cardiac looping (Riley et al., 1998; Riley et al., 2000). Interpretation of the phenotype of such chimeric embryos is complicated by possible extra-cardiac functions of Hand1 as well as the variable contribution of Hand1 null cells to extra-embryonic tissues.

Studies in chick and zebrafish embryos have also revealed potential functions of Hand genes in cardiac development. Exposure of chick embryos to antisense oligonucleotides for Hand1 and Hand2 together, but not separately, perturbs heart development at the looping stage, suggesting that these factors act redundantly (Srivastava et al., 1995). The zebrafish genome appears to encode only a single Hand gene, most closely related to Hand2, and mutations in this gene in hand2 knockouts, result in a dramatic reduction in ventricular precursors (Yelon et al., 2000). This phenotype is more severe than that observed in Hand2 knockout mice, possibly because of the lack of compensatory activity of a second Hand gene in this organism.

To determine the function of Hand1 in mouse heart development without complications from early lethality owing to extra-cardiac functions, we generated mice harboring a conditional Hand1 allele flanked by Cre recombinase loxP recognition sites, and deleted the gene specifically in the developing heart using a cardiac-specific α-myosin heavy chain (αMHC) promoter, or the Nkx2.5 cardiac enhancer to express Cre recombinase (Cre). Mice lacking myocardial
PCR genotyping
Tail and yolk sac DNA was isolated as previously described (McFadden et al., 2000). PCR reactions were used to detect Cre transgenes, and Hand1 and Hand2 knockout loci (Furulli et al., 1998; Srivastava et al., 1997). Briefly, 1 µl of tail or yolk sac DNA was used in a template in 25 µl PCR reactions using Promega Taq polymerase and 4 mM MgCl₂. Thermal cycle reactions were as follows: 2 minutes at 95°C, 30 cycles of 30 seconds at 95°C, 30 seconds at 55°C, 45 seconds at 72°C and a final 5 minute extension at 72°C. Reactions were visualized on 1% agarose gels in TAE. Primer sequences are available upon request.

RT-PCR
Left ventricles from E9.5 embryos were dissected and immediately frozen and stored in liquid nitrogen until embryo and yolk sac DNA was isolated and genotyped. Left ventricular tissue from eight mutant hearts was pooled and total RNA was isolated using Trizol reagent and standard protocols. Total LV RNA (150 ng) was used as a template for first strand cDNA synthesis using the Superscript first strand synthesis kit from Invitrogen. Five percent of the cDNA synthesis reaction was used as template for PCR reactions using Promega Taq polymerase to detect Hand1 transcripts. Transcripts for hypoxanthine phosphoribosyl transferase (HPRT) were detected as a control. Thermal cycles were as follows: 94°C for 2 minutes, 28 cycles of 94°C 30 for seconds, 52°C for 30 seconds, 72°C for 30 seconds and a final 5 minute extension at 72°C. Reactions were visualized on 1% agarose gels in TAE. Primer sequences are available upon request.

Histology
Embryos were harvested from timed matings and fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS). Following fixation, embryos were rinsed in PBS then dehydrated through graded ethanol and embedded in paraffin wax as previously described (Moller and Moller, 1994). Histological sections were cut and stained with Hematoxylin and Eosin, or nuclear Fast Red as previously described (Moller and Moller, 1994).

In situ hybridization
Section in situ hybridization was performed as described (Shelton et al., 2000). Whole-mount in situ hybridization was performed as previously described (Riddle et al., 1993). Plasmids for in situ probes have been previously described and were linearized and transcribed as follows: ANF, Xhol and T7 (Miller-Hance et al., 1993); connexin 40, Asp718 and T3 (Haefliger et al., 1992); Tbx5, SpeI and T7 (Bruneau et al., 1999); and mlc2V, BamHI and T7 (O’Brien et al., 1993). The coding regions of cited1 and cited2 were amplified as Clal-EcorI fragments and subcloned into pBSK. Both plasmids were linearized with XhoI and transcribed with T3 RNA polymerase. Hand2- and Hand1-coding regions were amplified as EcorI-XbaI fragments and cloned into pBSK. Both plasmids were linearized with EcorI and transcribed with T7.

β-Galactosidase staining
Embryos from timed matings were harvested and pre-fixed for 1-3 hours in 2% paraformaldehyde, 0.25% glutaraldehyde in PBS. Staining for β-gal activity was performed as previously described (McFadden et al., 2000).

TUNEL and immunohistochemistry
TUNEL staining was performed on paraffin wax embedded sections from E10.5 and E13.5 according to the Promega Fluorescein Apoptosis detection kit. Embryos were harvested at E11.5 and fixed overnight in 4% paraformaldehyde in PBS. Embryos were rinsed in PBS and equilibrated into 10% sucrose for 2 hours, followed by 30% sucrose overnight at 4°C. Embryos were transferred into freezing medium and frozen in isopentane and liquid nitrogen. Blocks were equilibrated to ~20°C and serially sectioned. Sections were stored at ~80°C until antibody staining. Antibody staining was performed as described (Frey et al., 2000). Primary anti-phospho histone H3 antibody was diluted 1:200 in 1% BSA in PBS.

Results
Generation of floxed Hand1 alleles
To create a conditional Hand1-null allele, we flanked the first exon of the mouse Hand1 gene with loxP sites by homologous recombination in ES cells (Fig. 1A). Our targeting strategy introduced a neomycin resistance cassette into the 5′-untranslated region of Hand1. Chimeric male mice generated from targeted ES cells transmitted the mutant allele through the germline yielding mice heterozygous for this Hand1neo-loxP allele. We bred heterozygous Hand1neo-loxP/+ mice to mice expressing the FLPe recombinase in the male germline (Rodriguez et al., 2000) in order to remove the neomycin resistance cassette. F1 progeny from these matings were genotyped by Southern blotting to detect the recombined allele (Hand1loxP) (Fig. 1A,B).

In order to determine if the Hand1loxP allele might function as a hypomorphic allele because of reduced expression, we bred mice heterozygous for this allele with mice heterozygous for the Hand1-null allele, referred to as Hand1lacZ, described previously (Furulli et al., 1998). Adult trans-heterozygous Hand1lacZ/+ mice bearing the two mutant alleles were overtly normal and fertile, suggesting that expression from the Hand1loxP allele was not significantly reduced. Homozygous Hand1loxPloxP mice were also phenotypically normal and fertile, and were used in subsequent breedings.

Early embryonic recombination in αMHC::Cre mice
In order to delete Hand1 specifically in the heart, we crossed Hand1loxPloxP females to Hand1lacZ/+ heterozygous male mice harboring a transgene that expresses Cre under the control of the α-myosin heavy chain (αMHC) promoter. These mice have been reported to express Cre in the embryonic and adult myocardium (Agah et al., 1997; Gaussen et al., 2002). In order to define precisely the onset of Cre-mediated recombination during embryogenesis, we intercrossed αMHC::Cre transgenic mice to ROSA26R indicator mice, which harbor a conditional lacZ allele that requires Cre-mediated recombination for expression (Soriano, 1999). β-Galactosidase (β-gal) activity from the ROSA26 locus was detected as early as E8.5 and by E9.0 expression was detected throughout the embryonic myocardium as well as in the outflow tract (Fig. 1C). At E11.5, both atrial and ventricular myocardium expressed high levels of lacZ. We did not detect lacZ expression elsewhere in these embryos.

To confirm that the Hand1loxP allele was efficiently recombined in vivo, we analyzed Hand1 expression in αMHC::Cre; Hand1loxPloxP/lacZ embryos at E10.5 by whole-mount in situ hybridization. As shown in Fig. 1D, Hand1 transcripts were specifically absent from the embryonic heart of these embryos, whereas expression of Hand1 was not affected in the branchial arches and lateral mesoderm. The absence of Hand1 transcripts in the LV by E9.5 was also confirmed by RT-PCR of cardiac RNA from αMHC::Cre; Hand1loxPloxP/lacZ embryos (Fig. 1E). These results demonstrated that efficient cardiac-specific removal of Hand1 transcripts occurred before E9.5.
Congenital heart defects resulting from cardiac deletion of Hand1

Genotyping of litters from intercrosses of \( \text{Hand1}^{\text{loxP/loxP}} \) to \( \alpha\text{MHC::Cre} \) revealed that offspring with the \( \alpha\text{MHC::Cre} \); \( \text{Hand1}^{\text{loxP/loxP}} \) genotype were born at Mendelian ratios (data not shown), but the majority became cyanotic and died within 3 days of birth. This suggested that loss of Hand1 in the embryonic myocardium resulted in perinatal lethality. Newborn mice of the \( \alpha\text{MHC::Cre} \); \( \text{Hand1}^{\text{loxP/loxP}} \) genotype nursed normally. At postnatal day 10 (P10), only 4% of offspring had the \( \alpha\text{MHC::Cre} \); \( \text{Hand1}^{\text{loxP/loxP}} \) genotype (Table 1). Only three mice of the \( \alpha\text{MHC::Cre} \); \( \text{Hand1}^{\text{loxP/loxP}} \) genotype survived to adulthood (representing less than 2% of offspring from the above intercross, or less than 1/10 of the predicted number of such mice).

Histological sections of P1-2 hearts revealed a spectrum of congenital heart defects in \( \alpha\text{MHC::Cre} \); \( \text{Hand1}^{\text{loxP/loxP}} \) mice (Fig. 2A), including membranous ventricular septal defects (VSDs) (Fig. 2A, part b), overriding aorta (Fig. 2A, part d), hyperplastic atrioventricular (AV) valves (Fig. 2A, parts c,f), and double outlet right ventricle (data not shown). All mice of this genotype displayed valve defects and 90% had VSDs. A somewhat smaller fraction of these mice had outflow tract defects.

To determine the time of onset of cardiac malformations in \( \text{Hand1} \) mutants, we harvested embryos from timed matings and analyzed cardiac morphology by histological sectioning (Fig. 2B). The left ventricular chamber of the mutants was reduced in size at E11.5 and 13.5 (Fig. 2B); however, chamber size appeared to recover by birth (Fig. 2A). Abnormalities in the early ventricular septum were noted as early as E10.5 (data not shown), and were obvious by E11.5 (Fig. 2B, compare a with
Embryonic cardiac ventricle expansion

Development and disease

b and c). At all developmental stages, the muscular ventricular septum of the mutant appeared thickened and disorganized. The embryonic AV endocardial cushions were also hyperplastic and, accordingly, the neonatal AV valves were thickened relative to those of wild type (e) and mutant (f) hearts. Arrows indicate the leaflets of the valves. (B) Hearts from wild-type (a,d) and Hand1lacZ/loxP; αMHC::Cre (mutant) (b,c,e,f) embryos at E11.5 (a-c) and E13.5 (d-f) were sectioned and stained with Hematoxylin and Eosin. The mutant hearts show poorly organized ventricular septa and left ventricular hypoplasia. Immature endocardial cushions are also seen in mutant hearts at E13.5. Arrowheads indicate the interventricular septum.

Cardiac defects generated with an Nkx2.5::Cre transgene

Expression of Hand1 is initially detected in the cardiac crescent at E7.75 (Cserjesi et al., 1995). Because the αMHC::Cre transgene does not direct high levels of Cre expression until after E8.5, it is possible that Hand1 has an important role earlier in cardiac development, in which case the cardiac phenotype we observed in αMHC::Cre; Hand1loxP/lacZ mice could reflect the incomplete deletion of Hand1 during early cardiogenesis. In an effort to eliminate cardiac Hand1 expression at an earlier stage, we expressed Cre recombinase fused to a nuclear localization signal (NLS) under control of Nkx2.5 regulatory sequences, which direct expression throughout the heart tube from the onset of cardiac commitment (Lien et al., 1999; Reecy et al., 1999). By E8.5, activity of these Nkx2.5 regulatory sequences is restricted to the developing ventricles and outflow tract. These sequences also direct expression in the thyroid primordium, and regions of the pharynx where Hand1 is not expressed.

Table 1. Genotypes of offspring at P10 from intercrosses of Hand1loxP/ to αMHC::Cre; Hand1loxZ/ mice

<table>
<thead>
<tr>
<th>Cardiac</th>
<th>KO/+</th>
<th>KO/KO</th>
<th>+/+</th>
<th>KO/+</th>
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<td>Number</td>
<td>56</td>
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<td>31</td>
<td>4</td>
<td>37</td>
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The Hand1loxP genotype is referred to as KO/+, and this genotype with the αMHC::Cre transgene as cardiac KO/KO. Mice heterozygous for the Hand1loxZ allele are referred to as +/+, and this genotype with the αMHC::Cre transgene as cardiac KO/+.
We generated six Nkx2.5::Cre transgenic lines, and crossed three lines into the ROSA26R heterozygous background to examine the efficiency and tissue-specificity of Cre-mediated recombination. All three lines exhibited a similar pattern of β-gal expression, which included heart, pharynx and a subset of cells within the liver (data not shown). We used a transgenic line (line 9) that directed the highest levels of recombination in the heart for all subsequent experiments. Efficient recombination was detected within the myocardium of Nkx2.5::Cre transgenic mice at the linear heart tube stage, and recombination occurred throughout the heart tube by E8.5 (Fig. 3A,B). Serial sections through stained E8.5 embryos revealed that the majority of cells in the right and left ventricular myocardium underwent recombination. At E10.5, the majority of cells within the LV were β-gal positive; however, some cells failed to express lacZ, presumably owing to a lack of Cre expression (data not shown). This may reflect downregulation of the Nkx2.5 cardiac enhancer in the LV at later stages of cardiac development (Lien et al., 1999) or mosaicism of transgene expression. At E12.5, high efficiency of Cre-mediated recombination was observed in the RV and LV. Interestingly, the outflow tract failed to undergo recombination (Fig. 3E), which may reflect contribution of a secondary heart field not derived from the cardiac crescent to the outflow tract myocardium (Kelly et al., 2001; Waldo et al., 2001).

We generated and analyzed Nkx2.5::Cre; Hand1loxP/lacZ mice. Like mutant mice bearing the αMHC::Cre transgene, these mice were viable until 2-4 days after birth, at which point they became cyanotic and died. We did not observe any Nkx2.5::Cre; Hand1loxP/lacZ mice at adulthood. However, we cannot rule out that a small fraction of such mice might survive, as was seen with the αMHC::Cre; Hand1loxP/lacZ genotype. Histological sectioning of mutants revealed similar congenital heart defects to those observed in αMHC::Cre; Hand1loxP/lacZ embryos (data not shown). This result suggested that removal of Hand1 transcripts before E8.5 did not cause embryonic lethality or exacerbate the phenotype observed in αMHC::Cre; Hand1loxP/lacZ embryos. Unless otherwise specified, we therefore used Nkx2.5::Cre mice.

![Fig. 3. Generation of Nkx2.5::Cre mice. (A,B) X-gal stained embryos showing staining in the linear heart tube (arrowhead) at E8.0 (A) and throughout the ventricular myocardium at E8.5 (B). (C,D) Nuclear Fast Red counterstained serial sections through the embryo in B. There are high levels of β-gal staining throughout the ventricular myocardium and in a subset of endocardial cells. (E) X-gal stained E12.5 embryo showing efficient ventricular recombination and minimal recombination in the outflow tract (oft) and atria (a). lv, left ventricle; rv, right ventricle; la, left atria; ra, right atria.](image1)

![Fig. 4. Abnormal cardiac morphogenesis in E10.5 embryos with compound mutations in Hand1 and Hand2. (A-F) E10.5 embryos stained for lacZ activity expressed from the Hand1loxP/lacZ allele. Left lateral view (A-C); frontal view (D-F). (G-I) Nuclear Fast Red counterstained transverse sections of the hearts of embryos in A-F. Genotypes of embryos are shown above each set of panels. There is severe reduction in Hand1-expressing cells in the LV and the hypoplastic ventricular chambers in H and I, and an absence of the interventricular groove and ventricular septation in I. lv, left ventricle; rv, right ventricle.](image2)
for the remainder of our analyses. Hereafter, we refer to the Hand1<sup>loxP</sup> allele in the presence of the Nkx2.5::Cre transgene as Hand1 cardiac-KO and the Hand1<sup>lacZ</sup> allele as Hand1 KO.

**Dose-sensitive requirements of Hand1 and Hand2 for left ventricular growth**

We next addressed the possibility that Hand1 and Hand2 act in a functionally redundant fashion during cardiac development. In contrast to Hand1, which is expressed specifically in the developing LV and conotruncus, Hand2 is expressed throughout the atrial and ventricular myocardium with highest levels of expression in the RV (Thomas et al., 1998). In Hand2 mutant embryos, the RV is hypoplastic, but the LV forms, albeit with fewer trabeculations (Srivastava et al., 1995). Because of their overlapping expression in the LV, it is possible that Hand2 may compensate for loss of Hand1 in this region of the developing heart. To address this possibility, we reduced the level of Hand2 expression by generating Hand1 cardiac-KO/KO; Hand2 KO/+ embryos from timed matings. Whereas Hand1 cardiac-KO/KO mice survived until birth and Hand2 KO/+ mice are normal, no embryos of the combined genotype were observed in litters harvested after E10.5. At E10.5, Hand1 cardiac-KO/KO; Hand2 KO/KO embryos were observed at Mendelian ratios, but appeared slightly delayed relative to Hand1 cardiac-KO/KO littermates. Expression of LacZ from the Hand1<sup>lacZ</sup> allele was decreased in the LVs of Hand1 cardiac-KO/KO embryos, and was further decreased in Hand1 cardiac-KO/KO; Hand2 KO/+ embryos, suggesting that expansion of the left ventricular chamber was

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**Fig. 5.** Abnormal cardiac morphogenesis in E9.0 embryos with compound mutations in Hand1 and Hand2. (A-L) E9.0 embryos stained for LacZ activity expressed from the Hand1<sup>lacZ</sup> allele. Left lateral view (A-F); frontal view of the heart (G-L). a, atrium; lv, left ventricle; rv, right ventricle; v, ventricular chamber. The asterisk in F shows the thin outflow tract present in the Hand1 cardiac-KO/KO; Hand2 KO/KO embryos. (M-X) Nuclear Fast Red counterstained transverse sections of embryos in A-L at anterior (M-R) and middle (S-X) levels of the heart. Genotypes of embryos are shown above each set of panels. Cardiac abnormalities increase in severity from left to right panels.
perturbed by the loss of Hand genes in a dose-sensitive manner (Fig. 4). Histological sectioning of Hand1 cardiac-KO/KO; Hand2 KO/+ embryos revealed a thin and poorly trabeculated myocardium (Fig. 4I). Thus, removal of one Hand2 allele in the absence of Hand1 generates an embryonic lethal defect in cardiac growth. We also tested whether removal of one Hand1 allele would exacerbate the Hand2 null phenotype. Indeed, Hand1 KO/+; Hand2 KO/KO embryos were morphologically delayed relative to Hand2 KO/KO embryos and died at E9.5, approximately 1 day earlier than Hand2 KO/KO embryos (Fig. 5E,K). Histological analysis of Hand1 KO/+; Hand2 KO/KO embryos showed evidence of only a single ventricle with a poorly developed myocardium, as is characteristic of Hand2 KO/KO embryos (Fig. 5Q,W). However, a prominent difference between Hand1 cardiac-KO/+; Hand2 KO/KO embryos and Hand2 KO/KO embryos was the diminished size of the ventricular lumen (Fig. 5, compare V and W) owing to an increase in the amount of cardiac jelly, a dense extracellular matrix between the endocardium and myocardium. Embryos homozygous for both mutant alleles (Hand1 cardiac-KO/KO; Hand2 KO/KO) displayed the most severe phenotype and did not survive beyond E9.0. The outflow tract and the atrial chamber were appropriately situated in these mutant embryos, indicating that looping of the heart tube had occurred (Fig. 5F), but there was only a single immature
ventricle and a common atrial chamber. Histological analysis of the double mutants’ hearts showed that some segments of the myocardial wall of the ventricular chamber presented an abnormal cellular morphology resembling mesenchymal cells rather than cardiomyocytes (data not shown). In addition, the trabeculae were poorly developed and the lumen of the ventricular chamber was abnormally narrow (Fig. 5R,X).

Dysregulation of ventricular gene expression in the absence of Hand genes

Several genes are expressed specifically along the outer curvature of the embryonic ventricles in patterns partially overlapping that of Hand1. Upregulation of these genes is thought to reflect the expansion of the chamber myocardium from the ventral surface of the more primitive linear heart tube myocardium (Christoffels et al., 2000). Nkx2.5-null embryos die at E10.5 from LV defects and fail to express Hand1 in the heart, which has led to the suggestion that Hand1 might act as an important downstream mediator of Nkx2.5 function during cardiac morphogenesis (Biben and Harvey, 1997; Lyons et al., 1995; Tanaka et al., 1999). Thus, we examined expression of potential Nkx2.5 target genes and other markers of chamber myocardium in Hand mutant embryos to determine whether they were sensitive to the level of Hand gene expression.

Atrial natriuretic factor (Anf; Nppa – Mouse Genome Informatics) is expressed in the embryonic LV in a pattern similar to that of Hand1. Expression of Anf was completely absent in the ventricles of both Hand1 cardiac-KO/KO; Hand2 KO/KO and Hand1 cardiac-KO/KO; Hand2 KO/KO (data not shown) embryos. Thus, left ventricular expression of Anf displayed a dose-dependency on the level of Hand1/2 expression, but was not absolutely dependent on Hand1.

Expression of ventricular myosin light chain 2 (Mlc2v; Myl2 – Mouse Genome Informatics) has also been reported to be downregulated or absent in Nkx2.5-null hearts (Lyons et al., 1995; Tanaka et al., 1999). However, we detected no change in Mlc2v expression in Hand1 cardiac-KO/KO embryos (Fig. 6A). Mlc2v transcripts were also detected in the ventricular remnant of Hand1 cardiac-KO/KO; Hand2 KO/KO double mutant embryos (Fig. 7A). We conclude that specification of ventricular cells occurs in the absence of Hand1 and Hand2, but ventricular expansion is perturbed. The expression of Anf and Mlc2v in Hand1 cardiac-KO/KO embryos also indicates that Nkx2.5 regulates these genes independently of Hand1.

The transcriptional co-activator Cited1 is expressed in a pattern that overlaps almost perfectly with that of Hand1 in the developing heart (Dunwoodie et al., 1998). Moreover, mice lacking the closely related factor Cited2 display congenital heart malformations similar to those we observed in Hand1 mutants (Bamforth et al., 2001). Interestingly, expression of
Cited1 was significantly downregulated in Hand1 mutant hearts (Fig. 6A). Expression of Cited2, which is broader than that of Cited1 during embryogenesis, was similar in wild-type and mutant hearts (data not shown).

Connexin 40 (Cx40; Gja5 – Mouse Genome Informatics), connexin 43 (Cx43; Gja1 – Mouse Genome Informatics), sarcoplasmic reticulum Ca\(^{2+}\) ATPase (Serca2a) and Tbx5 also show LV-restricted expression patterns. The expression of Serca2a was unaffected in Hand1 cardiac-KO/KO; Hand2 KO/+ embryos (data not shown). By contrast, expression of Cx40 was slightly decreased in Hand1 cardiac KO/KO embryos and was completely absent from the ventricular myocardium in Hand1 cardiac-KO/KO; Hand2 KO/+ embryos (Fig. 6A,B). It is notable in this regard that mice lacking Tbx5 also fail to express Anf and Cx40 (Bruneau et al., 2001). Therefore, to determine if Hand1 and Hand2 might act genetically upstream of Tbx5, we examined expression of Tbx5 in embryos lacking combinations of the Hand genes and found it to be unaffected (Fig. 6, Fig. 7B). We conclude that Hand1 and Hand2 regulate Cx40 and Anf expression through mechanisms independent of Tbx5 transcription.

**Discussion**

The Hand genes have been shown to display dynamic and highly specific expression patterns during heart development in mouse, chick, frog and zebrafish embryos (Cserjesi et al., 1995; Srivastava et al., 1995; Angelo et al., 2000; Yelon et al., 2000). In the mouse, Hand2 is required for right ventricular development (Srivastava et al., 1997), but potential cardiac functions of Hand1 have remained elusive owing to its required role in the placenta, which causes early embryonic lethality of Hand1 null embryos (Firulli et al., 1998; Riley et al., 1998). The findings of the present study reveal specific functions of Hand1 in growth and maturation of the heart, distinct from those of Hand2. In addition, analysis of Hand1/2 compound mutant mice demonstrates that Hand transcription factors act in a gene dose-sensitive manner to regulate expansion of the ventricular chambers and expression of specific sets of cardiac genes during embryonic development. A model for the roles of mouse Hand genes in ventricular development is shown in Fig. 8.

**Ventricular septal defects in Hand1 cardiac KO mice**

Cardiac-specific deletion of Hand1 resulted in a spectrum of heart abnormalities leading to death in the perinatal period. The most common defects observed in embryos and neonates were VSDs, AV valve abnormalities, and outflow tract abnormalities. VSDs are the most prevalent congenital heart defect in humans (Hoffman, 1995), and have been observed in a variety of mouse mutants (Srivastava, 2001). Regions of the AV cushions contribute to both the atrial and ventricular septa, and alterations in AV cushion remodeling result in septal defects involving the membranous ventricular septum (Bartram et al., 2001; Bamforth et al., 2001; Eisenberg and Markwald, 1995). It is therefore likely that defects in AV cushion remodeling contribute, at least in part, to the VSDs observed in Hand1 mutants. However, in addition to AV cushion abnormalities, Hand1 cardiac KO mice exhibit a thickened, disorganized muscular septum at all stages of embryonic development. This and the fact that we occasionally observe defects in the muscular ventricular septum suggest that growth of septal myocytes or positioning of the interventricular septum are abnormal in Hand1 mutants.

Hand1 expression is excluded from all but a small subpopulation of septal myocytes, suggesting that it plays a non-cell autonomous role in definition of the septal boundary, or that septal defects are secondary to abnormal growth and morphogenesis of the LV. A recent study provides evidence that Hand1 is indeed an important regulator of the interventricular boundary (Toghi et al., 2004). Using homologous recombination, Hand1 was targeted to the Mlc2v locus, which is expressed throughout the ventricular and septal myocardium. Hand1 knock-in mice died at midgestation and completely lacked an interventricular septum. Thus, overexpression of Hand1 eliminates the interventricular septum, while cardiac deletion of Hand1 causes expansion of this region of the heart.

**Valve defects resulting from cardiac Hand1 deletion**

Embryos that lack cardiac expression of Hand1 also displayed abnormally thickened AV valves and hyperplastic endocardial cushions. The endocardial cushion malformations in these mutant mice are interesting because the αMHC::Cre transgene does not direct Cre expression in the endocardial cushions or cardiac valves (Agah et al., 1997; Guassman et al., 2002). This suggests that Hand1 regulates a myocardium-derived signal that controls endocardial cushion morphogenesis. Crosstalk between the myocardium and endocardium is well documented and has been shown to involve BMP- and TGFβ-mediated signals (Brown et al., 1999; Eisenberg and Markwald, 1995; Guassman et al., 2002; Kim et al., 2001). Mice that lack TGFβ2 or Smad6, a BMP effector, also exhibit hyperplastic cardiac valves (Galvin et al., 2000; Bartram et al., 2001). However, Bmp2, Bmp4, Smad6, Smad7 and Tgbf expression is unaltered in Hand1 mutants (data not shown), suggesting that Hand1 regulates other signaling pathways. It should also be noted that early myocardial function profoundly influences endocardial cushion development (Bartman et al., 2004). Thus, abnormalities in cardiac contractility or morphogenesis in embryos lacking cardiac Hand1 expression could indirectly influence the formation of cardiac cushions.
Redundancy of mammalian Hand genes

The cardiac phenotype resulting from cardiac deletion of Hand1 was much less severe than that of Hand2 mutant embryos in which the entire right ventricular region of the heart is absent (Srivastava et al., 1997). These differences in ventricular phenotypes are likely to reflect important distinctions in the expression patterns of Hand1 and Hand2. Hand1 is expressed specifically in the outer curvatures of the embryonic LV, RV and outflow tract. By contrast, Hand2 is expressed throughout the left and right ventricular chambers, although its expression is highest in the RV (Srivastava et al., 1997; Biben and Harvey, 1997). Therefore, in the absence of Hand1, residual Hand2 expression in the LV and outflow tract may partially compensate for the loss of Hand1. By contrast, in the absence of Hand2 there is a more complete lack of Hand factors in the presumptive RV (Srivastava et al., 1997).

The genome of the zebrafish encodes a single Hand gene, and loss-of-function mutations (hands off) result in a near complete absence of ventricular precursors. This has led to the speculation that mouse Hand1 and Hand2 act redundantly during ventricular differentiation (Yelon et al., 2000). Our results are consistent with this notion. Removal of one copy of the Hand2 gene in the setting of cardiac-specific deletion of Hand1 exacerbated the Hand1 cardiac phenotype and resulted in embryonic lethality at midgestation; removal of both Hand2 genes caused severe ventricular hypoplasia and lethality at yet an earlier developmental stage. However, the cardiac phenotype of our Hand1 cardiac KO/KO; Hand2 KO/KO is less severe than that of zebrafish hands off mutants. Although these results must be interpreted in light of the technical limitations of Cre-mediated gene deletion, this may also suggest that mammalian Hand genes have acquired unique functions during the evolution of the four-chambered heart.

Hand genes regulate ventricular expansion

The morphogenesis of the single-chamber embryonic heart into the adult four-chamber heart is crucially dependent on the expansion, or ‘ballooning’ of the outer curvature of the right and left ventricular chambers (Christoffels et al., 2000). Expression of Hand1 along the outer curvature of the LV and outflow tract is consistent with a role in the expansion of chamber myocardium. The expression patterns of several genes mark the expansion of this chamber myocardium from the outflow tract is consistent with a role in the expansion of ventricular myocardium. The exquisite sensitivity of Hand1 mutant hearts to Hand2 gene dose underscores the crucial role of these genes during ventricular expansion.

What mechanism(s) might account for the severe ventricular hypoplasia seen in mice lacking cardiac expression of Hand1 and Hand2? Because we did not detect significant differences in apoptosis or proliferation of ventricular myocytes in wild-type and double Hand mutant embryos, it is unlikely that abnormalities in these events account for the severe deficiency of ventricular myocytes in the mutant. Therefore, we speculate that the absence of both Hand genes results in a deficiency in specification of cardiac myocytes at an early stage of cardiogenesis. A similar mechanism has been proposed to account for the lack of cardiomyocytes in the hands off mutant (Yelon et al., 2000).

The expression pattern of Hand1 in the developing heart is nearly identical to that of Cited1, which encodes a transcriptional co-activator (Biben and Harvey, 1997; Dunwoodie et al., 1998). The downregulation of Cited1 in Hand1 mutant embryos suggests that Hand1 acts upstream of Cited1 during cardiac development. It is notable, in this regard, that cited1 and Hand1 are also co-expressed in trophoblastic tissues of the placenta, and mice lacking either gene display lethal defects in placental development (Rodriguez et al., 2004; Riley et al., 1998; Firulli et al., 1998). Analysis of heart morphology has not been described in Cited1 mutants (Rodriguez et al., 2004), but mice lacking Cited2 display numerous congenital heart defects that overlap with those observed in Hand1 cardiac KO animals (Bamforth et al., 2001). However, we detected no difference in Cited2 expression between wild-type and Hand1 mutant mouse lines (data not shown). It remains possible that subtle cardiac defects are indeed present in Cited1-null mice, or differences in genetic background between Hand1 and Cited1 mutant mouse lines account for the lack of obvious cardiac defects in Cited1-null mice. Regardless, it is likely that downstream targets in addition to Cited1 contribute to the heart defects observed in Hand1 cardiac KO animals.

The relationship between Hand1 and Nkx2.5

In mice lacking Nkx2.5, the left ventricular chamber fails to expand following cardiac looping, and expression of several markers of cardiac differentiation is reduced throughout the remaining myocardium (Lyons et al., 1995; Tanaka et al., 1999; Yamagishi et al., 2001). Interestingly, Hand1 expression is abolished in the hearts of Nkx2.5 mutant embryos (Biben and Harvey, 1997). Therefore, it has been proposed that loss of Hand1 contributes to abnormal cardiac morphogenesis of Nkx2.5 mutant hearts (see Fig. 8). The data presented here suggest that lack of Hand1 is not solely responsible for left ventricular hypoplasia in Nkx2.5-null mice. First, loss of Hand1 expression in the early heart tube results in only a modest and transient decrease in size of the left ventricular chamber in the embryo, much less severe than in Nkx2.5 mutants. Second, in contrast to Nkx2.5 null mice, Anf and Mlc2v are expressed at high levels in the LV of Hand1 mutants. Both Anf and Mlc2v are also normally expressed in Hand1+/+Zic2+/+Zic2 null mice (Firulli et al., 1998), demonstrating that expression of these markers in Hand1 conditional knockout mice is not due to inefficient or delayed excision of Hand1. In addition, markers of the left ventricular chamber, including Tbx5 and Cx40, are expressed normally in the absence of Hand1.
We previously showed that mice deficient in Nkx2.5 and Hand2 formed only a single cardiac chamber, molecularly defined as the atrium (Yamagishi et al., 2001). By contrast, in the absence of cardiac Hand expression, ventricular cardiomyocytes are evident as shown by expression of Mlc2v, however ballooning of the ventricular chamber is abrogated. The less severe phenotype of the Hand1/Hand2 mutant again suggests that Nkx2.5 regulates genes in addition to Hand1 and that the lack of Hand1 expression is only partially responsible for cardiac defects and embryonic lethality observed in Nkx2.5-null mice.

**Contributions of primary and secondary heart fields to the developing heart**

It is interesting to consider the roles of Hand1 and Hand2 in the context of the contributions of the primary and secondary heart fields to the developing heart. The primary heart field is believed to give rise to the atria and left ventricular chambers, while the secondary (or anterior) heart field contributes primarily to the outflow tract and right ventricular region of the heart (reviewed by Kelly et al., 2001; Kelly and Buckingham, 2002; Abu-Issa et al., 2004) (see also Cai et al., 2003). The absence of Hand2 results in the deletion of the right ventricular regions of the heart (Srivastava et al., 1997), suggesting that it is an essential component of the pathway for development of the secondary heart field. The cardiac phenotype of Nkx2.5 mutant embryos is complementary to that of Hand2 mutants, i.e. a lack of the left ventricular region (Lyons et al., 1995; Tanaka et al., 1999). Consistent with the idea that Hand2 and Nkx2.5 regulate growth of complementary regions of the heart, mice lacking both genes form only a primitive cardiac rudiment (Yamagishi et al., 2001). It is notable that the zebrafish heart has only a single ventricle and no evidence of a secondary heart field. The presence of only a single Hand gene in that organism raises the possibility that a primordial Hand gene may have been duplicated during evolution such that each Hand gene fell under separate temporal and spatial control as a means of generating two heart fields that contribute to the different ventricular chambers.

**Implications**

The types of cardiac abnormalities observed in mice lacking either Hand1 or Hand2 are reminiscent of congenital heart defects in humans. Most congenital heart defects in humans are due to genetic or environmental modifiers. By contrast, most cardiac defects studied in the mouse arise owing to homozygous gene deletion. Given the diversity of cardiac abnormalities that can result from Hand gene mutations (ventricular hypoplasia, VSDs, valve defects, outflow tract abnormalities), we anticipate that the Hand genes will prove to be crucial for heart development and congenital heart disease in humans.

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