Developmental Biology 239, 190–203 (2001)
doi:10.1006/dbio.2001.0417, available online at http://www.idealibrary.com on IDEAL®

The Combinatorial Activities of Nkx2.5 and dHAND Are Essential for Cardiac Ventricle Formation

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Nkx2.5/Csx and dHAND/Hand2 are conserved transcription factors that are coexpressed in the precardiac mesoderm and early heart tube and control distinct developmental events during cardiogenesis. To understand whether Nkx2.5 and dHAND may function in overlapping genetic pathways, we generated mouse embryos lacking both Nkx2.5 and dHAND. Mice heterozygous for mutant alleles of *Nkx2.5* and *dHAND* were viable. Although single *Nkx2.5* or *dHAND* mutants have a morphological atrial and single ventricular chamber, *Nkx2.5*$^{-/-}$ *dHAND*$^{-/-}$ mutants had only a single cardiac chamber which was molecularly defined as the atrium. Complete ventricular dysgenesis was observed in *Nkx2.5*$^{-/-}$ *dHAND*$^{-/-}$ mutants; however, a precursor pool of ventricular cardiomyocytes was identified on the ventral surface of the heart tube. Because *Nkx2.5* mutants failed to activate eHAND expression even in the early precardiac mesoderm, the *Nkx2.5*$^{-/-}$ *dHAND*$^{-/-}$ phenotype appears to reflect an effectively null state of dHAND and eHAND. Cell fate analysis in *dHAND* mutants suggests a role of *HAND* genes in survival and expansion of the ventricular segment, but not in specification of ventricular cardiomyocytes. Our molecular analyses also revealed the cooperative regulation of the homeodomain protein, Irx4, by Nkx2.5 and dHAND. These studies provide the first demonstration of gene mutations that result in ablation of the entire ventricular segment of the mammalian heart, and reveal essential transcriptional pathways for ventricular formation. © 2001 Academic Press

Key Words: Nkx2.5; dHAND; transcriptional regulation; cardiac development; congenital heart disease.

INTRODUCTION

Embryonic ventricular cardiomyocytes undergo rapid proliferation, but lose the ability to substantially expand in number postnatally and are thereafter unable to repopulate areas of myocardial loss or injury. Congenital heart defects involving hypoplasia of the right or left ventricle account for 25% of all mortality from congenital heart disease in children and may be the result of defects in expansion of a precursor pool of ventricular cardiomyocytes. Interestingly, the atrial chambers and remaining ventricular chamber in such conditions are relatively normal, suggesting that separable regulatory pathways may govern cardiac chamber-specific development (Srivastava, 2001). For these reasons, there has been much interest in understanding the regulatory networks that control segmental patterning and growth of distinct cardiac chambers.

Numerous *cis*-regulatory elements are known to direct transcription in a chamber-specific fashion and may contribute to segmental development of the heart (Ross *et al.*, 1996; Li *et al.*, 1996; Kelly *et al.*, 1995; Xavier-Neto *et al.*, 1999; Kuo *et al.*, 1999). More recently, a transcriptional basis for segmental cardiac development has begun to emerge (reviewed in Srivastava and Olson, 2000; Fishman and Olson 1997; Fishman and Chien, 1997). For example, a homeodomain protein belonging to the Iroquois family, Irx4, directs ventricular-specific gene expression (Bao *et al.*, 1999) and the orphan nuclear receptor, COUP-TF (chicken ovalbumin upstream promoter-transcription factor) II, is required for atrial, but not ventricular development (Pereira *et al.*, 1999). Members of the HRT family of hairy-related

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basic helix–loop–helix (bHLH) proteins, also known as Hesr, Hey, CHF, or Gridlock (Nakagawa *et al.,* 1999; Kokubo *et al.,* 1999; Leimeister *et al.,* 1999; Chin *et al.,* 2000; Zhong *et al.,* 2000), are expressed in a complementary fashion in the embryonic heart, with HRT1 in the atria and HRT2 in the ventricles, respectively (Nakagawa *et al.,* 1999; Leimeister *et al.,* 1999).

dHAND/Hand2 and eHAND/Hand1 are closely related members of another bHLH subclass of transcription factors (Srivastava, 1999) that are highly conserved across species and are required for heart development in the chick and mouse. In chick embryos, dHAND and eHAND are coexpressed throughout the heart tube before, during, and after cardiac looping and display a degree of functional redundancy (Srivastava *et al.,* 1995). In mice, dHAND expression becomes specifically enhanced in the right ventricle after cardiac looping (Srivastava *et al.,* 1997; Thomas *et al.,* 1998a). Mouse eHAND is expressed in left ventricular precursors but, unlike chick, is completely excluded from the right ventricular segment as early as the straight heart tube stage (Srivastava *et al.,* 1997; Biben and Harvey, 1997). Consistent with the expression patterns of dHAND and eHAND, mouse embryos lacking dHAND display specific hypoplasia of the right ventricle soon after cardiac looping (Srivastava *et al.,* 1997). The less severe left ventricular phenotype may be the result of partial compensation by eHAND in the left ventricle. *eHAND*-null embryos die early from placental insufficiency precluding detailed cardiac analyses (Firulli *et al.,* 1998; Riley *et al.,* 1998); however, generation of *eHAND*-null chimeric embryos suggests a role for eHAND in left ventricular development (Riley *et al.,* 2000).

In addition to the left–right polarity in cardiac ventricular gene expression, a dorsal–ventral polarity exists as early as embryonic day (E) 8.0 (straight heart tube stage) in the mouse. The ventral surface of the early heart tube becomes the outer curvature of the looped heart tube and undergoes rapid proliferation and develops trabeculations. In contrast, the dorsal surface becomes the inner curvature, and myocardial cells ultimately evacuate this region allowing for the extensive remodeling of the looping heart that is necessary for proper alignment of the cardiac inflow and outflow tracts (Mjaatvedt *et al.,* 1998). A model has been proposed in which cardiac chambers form by virtue of rapid "ballooning" growth along the outer curvature of segments of the heart tube (de Jong *et al.,* 1997; Christoffels *et al.,* 2000). Interestingly, eHAND is expressed on the ventral surface of the straight heart tube and later along the outer, but not the inner, curvature of the looped heart (Thomas *et al.,* 1998a, Biben and Harvey, 1997). The significance of this differential pattern of gene expression has yet to be explored.

Nkx2.5/Csx, a member of the Nk-2 family of homeodomain proteins, is another highly conserved transcription factor (Harvey, 1996; Evans, 1999) that was identified as a mammalian orthologue of *Drosophila* tinman (Lints *et al.,* 1993; Komuro and Izumo, 1993; Bodmer, 1993). Heterozygous mutations in human *NKX2.5* or mouse *Nkx2.5* result in a spectrum of congenital heart defects, suggesting that this transcription factor contributes to diverse cardiac developmental pathways in mammals (Schott *et al.,* 1998; Benson *et al.,* 1999; Biben *et al.,* 2000). In mice homozygous null for *Nkx2.5*, the heart tube forms, but a morphological defect at the looping stage occurs, and several cardiac genes, including eHAND, are downregulated in the *Nkx2.5*-null ventricle (Lyons *et al.,* 1995; Tanaka *et al.,* 1999).

A challenge in cardiovascular biology is to develop an understanding of the hierarchical and combinatorial networks through which critical transcription factors control the form and function of the developing heart (Sucov, 1998). Previous studies have shown that neither Nkx2.5 nor dHAND regulate one another (Srivastava *et al.,* 1997; Biben and Harvey, 1997). To determine whether Nkx2.5 and the HAND proteins may function in common or parallel pathways during cardiac development, we generated mouse embryos heterozygous and homozygous null for both *Nkx2.5* and *dHAND*. Double heterozygous mice were apparently normal. In embryos homozygous null for both *Nkx2.5* and *dHAND*, myocardial differentiation was unaffected. However, embryos lacking both Nkx2.5 and dHAND formed only a single cardiac chamber that was molecularly defined as the atrium. Complete ventricular dysgenesis occurred, although a small pool of ventricular-specific cells was detected along the ventral surface of the atrial chamber. *In vivo* analysis of eHAND expression revealed that *Nkx2.5* mutants failed to activate eHAND expression even in the early cardiac crescent, suggesting that the *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ phenotype reflected an effectively null state of dHAND and eHAND. In the absence of any *HAND* gene expression in the right ventricle, right ventricular cardiomyocytes were specified but failed to expand in number and underwent programmed cell death. Finally, our results reveal a cooperative effect of Nkx2.5 and dHAND on regulation of the ventricular-specific homeodomain protein, Irx4. These results suggest that Nkx2.5 and dHAND are not essential for myocardial differentiation, but together are required for expansion of a precursor population of ventricular cardiomyocytes during mouse heart development.

MATERIALS AND METHODS

Generation of Nkx2.5$^{-/-}$*dHAND*$^{-/-}$ *Embryos*

Mice heterozygous or homozygous for an *Nkx2.5* mutation (Lyons *et al.,* 1995) or *dHAND* mutation (Srivastava *et al.,* 1997) were generated and genotyped as described previously on a C57L/B6 background. Double heterozygous mice (*Nkx2.5*$^{+/-}$*dHAND*$^{+/-}$) were intercrossed to generate mice with all possible combinations of *Nkx2.5* and *dHAND* gene disruptions. Pregnant mothers were sacrificed and their uteri dissected to harvest E8.0–E10.5 embryos, which were fixed in 4% paraformaldehyde.

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TABLE 1
Genotype of Embryos from *Nkx2.5*$^{+/-}$ *dHAND*$^{+/-}$ Intercrosses

	Nkx2.5	+/+	+/+	+/+	+/−	+/−	+/−	−/−	−/−	−/−	
Genotype	*dHAND*	+/+	+/−	−/−	+/+	+/−	−/−	+/+	+/−	−/−	Total
E8.5	Observed	1	7	3	4	6	4	1	5	2	33
	Expected	2	4	2	4	8	4	2	4	2	
E9.5	Observed	11	21	6	18	28	12	6	14	7	123
	Expected	8	15	8	15	31	15	8	15	8	
E10.5	Observed	5	8	2	11	9	3	1	1	0	40
	Expected	3	5	3	5	10	5	3	5	3	
Expected Ratio		1/16	1/8	1/16	1/8	1/4	1/8	1/16	1/8	1/16	

Breeding and Genotyping of Transgenic Mice

To generate transgenic mice in a *dHAND*-null background, male mice heterozygous for the *MLC2V-lacZ* transgene (Ross *et al.*, 1996) were mated to *dHAND* heterozygous females and offspring back-crossed to generate embryos with the *MLC2V-lacZ* transgene in the *dHAND*-null background. Similar crosses were performed with *Nkx2.5* heterozygous mice and mice heterozygous for a *lacZ* insertion into the *eHAND* genomic locus (Firulli *et al.*, 1998). Mothers were sacrificed and their uteri dissected to harvest E7.5–E9.5 embryos. Genotyping was performed as described previously (Ross *et al.*, 1996; Firulli *et al.*, 1998). β-Galactosidase staining to detect lacZ expression was performed as described previously (Yamagishi *et al.*, 2000).

Histology

Wild-type, *Nkx2.5*, and/or *dHAND* mutant embryos were embedded in paraffin, and serial transverse sections were obtained at 7-μm intervals. Paraffin was cleared with xylene, and sections stained with hematoxylin and eosin.

Whole-Mount and Section in Situ Hybridization

Whole-mount RNA *in situ* hybridizations for myosin light chain (MLC)1A, MLC2A, MLC2V, and eHAND were performed by using digoxigenin-labeled antisense riboprobes as described previously (Yamagishi *et al.*, 2000). *In situ* hybridization to mouse embryo sections was performed as described previously (Lu *et al.*, 1998). ^{35}S-labeled antisense riboprobe was synthesized with T3, T7, or SP6 RNA polymerase (MAXIscript; Ambion Inc., Austin, TX) from MLC2V, COUP-TFII, Tbx5, HRT1, HRT2, or Irx4 cDNA. Hybridization was performed on paraffin-embedded sections of E8.0–E9.25 mouse embryos. Sources of DNAs for making probes were as follows: MLC1A, MLC2A, MLC2V, eHAND (Srivastava *et al.*, 1997; Firulli *et al.*, 1998), COUP-TFII (Pereira *et al.*, 1999), Tbx5 (Bruneau *et al.*, 1999), HRT1 and HRT2 (Nakagawa *et al.*, 1999), Irx4 (Bruneau *et al.*, 2000).

TUNEL Assay for Apoptosis and BrdU Incorporation

To visualize apoptotic nuclei *in situ*, transverse sections of E8.25 wild-type, *dHAND*$^{-/-}$, *Nkx2.5*$^{-/-}$, or *Nkx2.5*$^{-/-}$ *dHAND*$^{-/-}$ hearts were subjected to terminal transferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay as previously described (Thomas *et al.*, 1998b). Cell proliferation assays on E8.25 wild-type, *dHAND*$^{-/-}$, *Nkx2.5*$^{-/-}$, or *Nkx2.5*$^{-/-}$ *dHAND*$^{-/-}$ embryos were performed by intraperitoneal injection of bromo-deoxyuridine (BrdU) at a concentration of 500 mg/kg. After a 2-h pulse, embryos were removed and fixed in 4% paraformaldehyde. BrdU immunofluorescence was performed on transverse sections by incubating in BrdU primary antibody (1:50) followed by incubation in a secondary antibody (goat anti-mouse IgG-Liss. Rhodamine).

RESULTS

Nkx2.5$^{-/-}$ *dHAND*$^{-/-}$ Embryos Die between E9.5 and 10.5

To explore possible genetic interactions between *Nkx2.5* and *dHAND* during embryogenesis, *Nkx2.5* and *dHAND* heterozygous mice were intercrossed to generate double heterozygotes (*Nkx2.5*$^{+/-}$ *dHAND*$^{+/-}$). *Nkx2.5*$^{+/-}$ *dHAND*$^{+/-}$ mice grew normally, were fertile, and had a normal life span, although subtle morphological or physiological abnormalities could not be ruled out. Double heterozygotes were intercrossed to generate embryos homozygous null for both *Nkx2.5* and *dHAND* (*Nkx2.5*$^{-/-}$ *dHAND*$^{-/-}$). Twenty-eight litters from the heterozygote intercrosses were examined between E8.25 and E10.5 (Table 1). At E10.5, no embryos homozygous null for both *Nkx2.5* and *dHAND* were observed, while embryos homozygous for each individual gene were observed with marked growth retardation. At E9.5, genotypes of embryos approximated Mendelian inheritance of both *Nkx2.5* and *dHAND* mutant alleles, suggesting that *Nkx2.5*$^{-/-}$ *dHAND*$^{-/-}$ mutants die between E9.5 and 10.5, one-half to one day earlier than embryos in which each single gene was disrupted. Little variability was observed in the cardiac phenotype of double homozygous mutant embryos (described below), indicating full phenotypic penetrance.

Cardiac Defects in *Nkx2.5*$^{-/-}$ *dHAND*$^{-/-}$ Embryos

Precardiac mesodermal cells form a crescent shape in the anterior part of the E7.75 embryo and subsequently converge along the midline to form a beating heart tube (Yutzey

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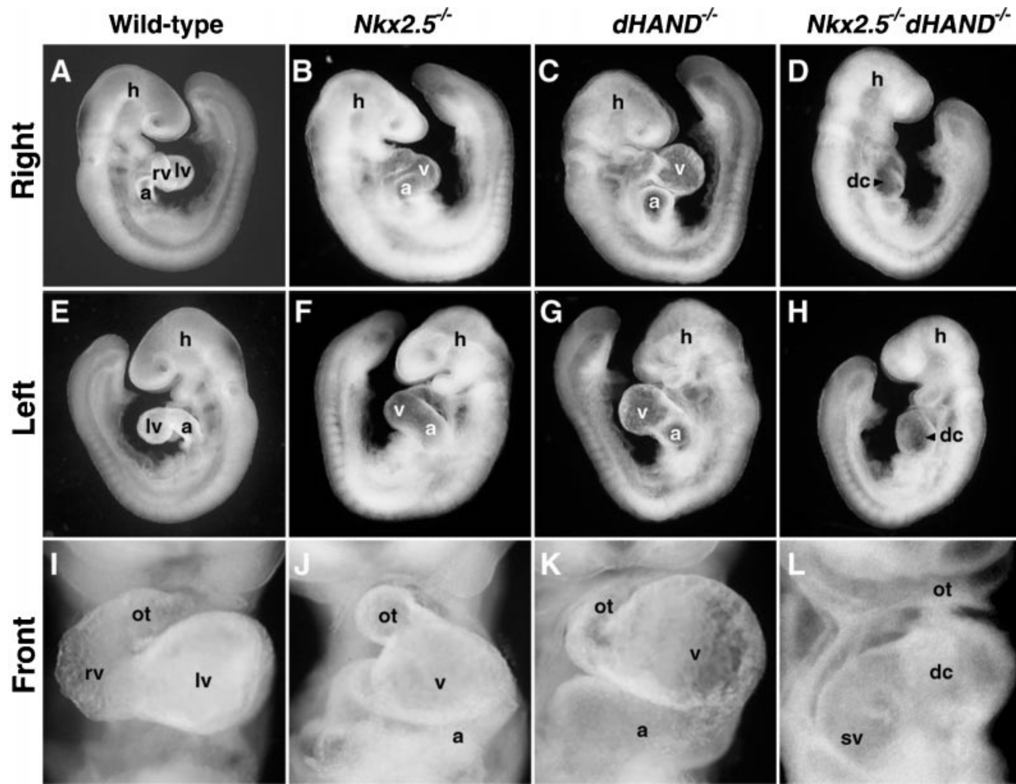

FIG. 1. Cardiac defects in *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ embryos. E9.25 embryos of wild-type (A, E, I), *Nkx2.5*$^{-/-}$ (B, F, J), *dHAND*$^{-/-}$ (C, G, K), and *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ (D, H, L) embryos are shown in right lateral (A–D), left lateral (E–H), and frontal (I–L) views. Although ventral protrusion of a ventricular segment (v) was obvious in the wild-type, *Nkx2.5*$^{-/-}$ and *dHAND*$^{-/-}$ embryos, only a single dorsally located chamber (dc) was present in the *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutants. Frontal views focus on the heart. a, atria; h, head; ot, outflow tract; rv, right ventricle; lv, left ventricle; sv, sinus venosae.

and Bader, 1995). Cardiac segmentation becomes apparent at the linear heart tube stage with the primitive right and left ventricles forming most of the fused anterior heart tube and the future atrial regions lying in the more posteriorly located sinus venosae. Subsequently, by virtue of rightward looping of the heart tube, the future right and left ventricles adopt their left–right orientation and become morphologically distinct. The atrial segment initially moves dorsal to the ventricles and the atrio-ventricular junction lies on the left side of the embryo (Figs. 1A, 1E, and 1I).

Previous studies reported that the linear heart tube failed to undergo cardiac looping in *Nkx2.5*$^{-/-}$ mutants (Lyons *et al.,* 1995). Our detailed analyses show that, while looping was incomplete, the outflow tract of *Nkx2.5*$^{-/-}$ mutants was always located on the right side, and the atrial region was located dorsally and on the left side, indicating that rightward looping of the heart tube was initiated in *Nkx2.5*$^{-/-}$ mutants (Figs. 1B, 1F, and 1J). Heart development was arrested during the process of looping, and there grossly appeared to be only a single segment of ventricle and a

FIG. 2. Histologic analysis of *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ hearts. Transverse sections of wild-type (A, E, I), *Nkx2.5*$^{-/-}$ (B, F, J), *dHAND*$^{-/-}$ (C, G, K), and *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ (D, H, L) embryos at E9.25 are shown at the anterior (A–D), middle (E–H), and posterior (I–L) levels of the heart. The segmentation of chambers into atria (a) and ventricles (v) is demarcated by arrowheads in (A), (E), (F), and (G). There was only a dorsal chamber (dc) apparent in the *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutant at any level of section. An accumulation of cells was observed in the ventral region of the heart (H, bracket). lv, left ventricle; ot, outflow tract; rv, right ventricle; sv, sinus venosae.

FIG. 3. Expression of MLC2A in *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ embryos. Expression of MLC2A, a marker for myocardial differentiation, was examined in wild-type (A), *Nkx2.5*$^{-/-}$ (B), *dHAND*$^{-/-}$ (C), and *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ (D) E9.25 embryos by whole-mount *in situ* hybridization. Left lateral views are shown. Expression was seen in ventricular (v) and atrial (a) chambers in each single mutant, and in the dorsal chamber (dc) of double mutants. lv, left ventricle; pa, pharyngeal arch; sv, sinus venosae.

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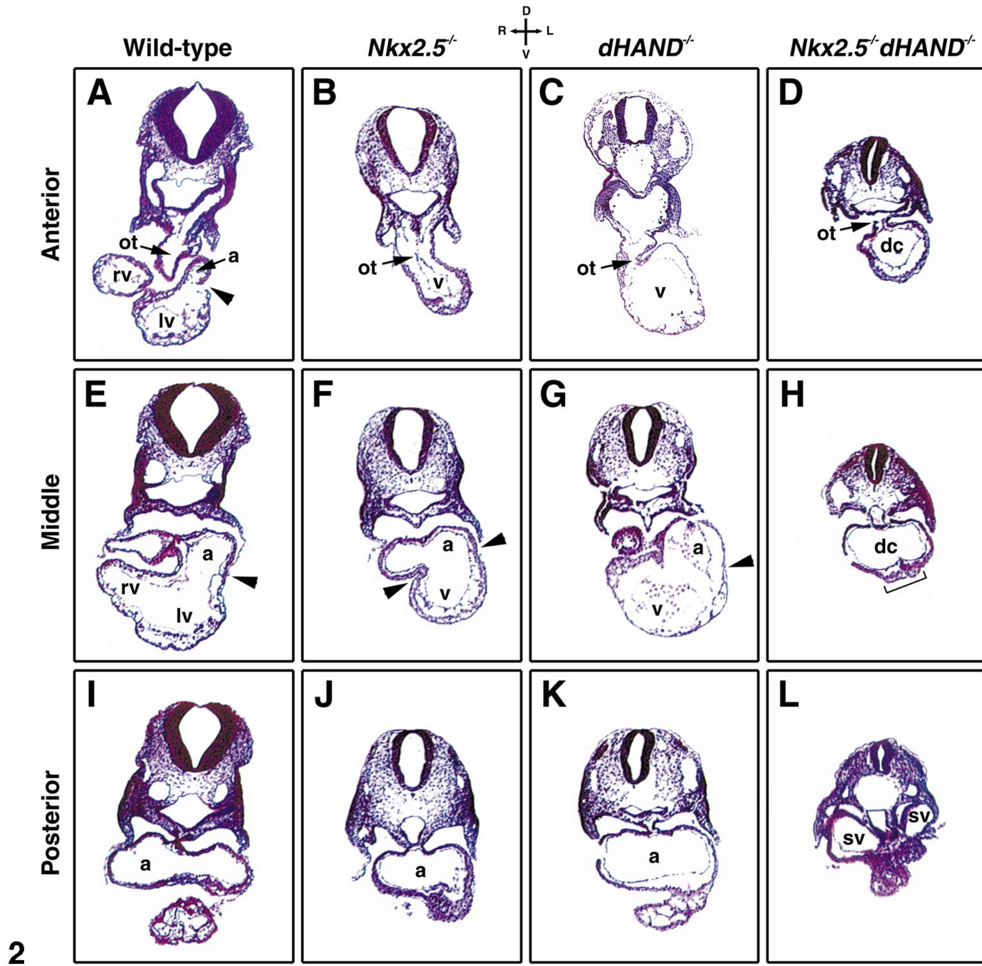

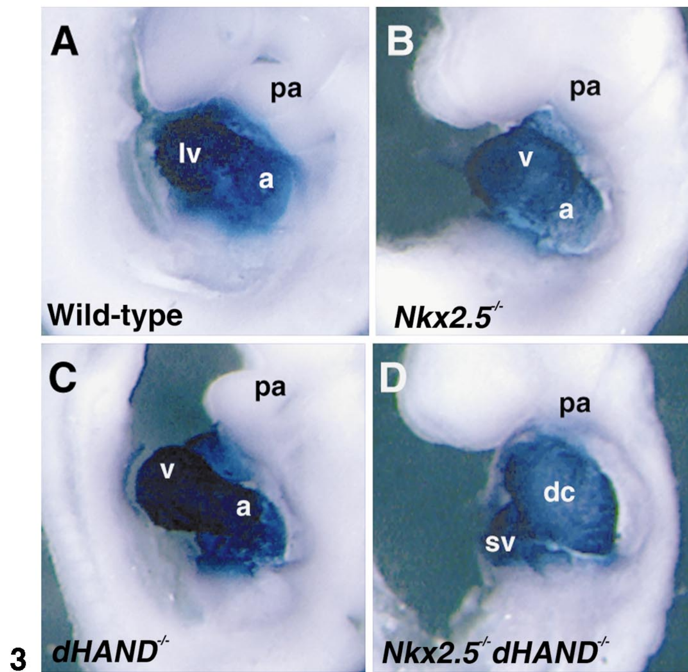

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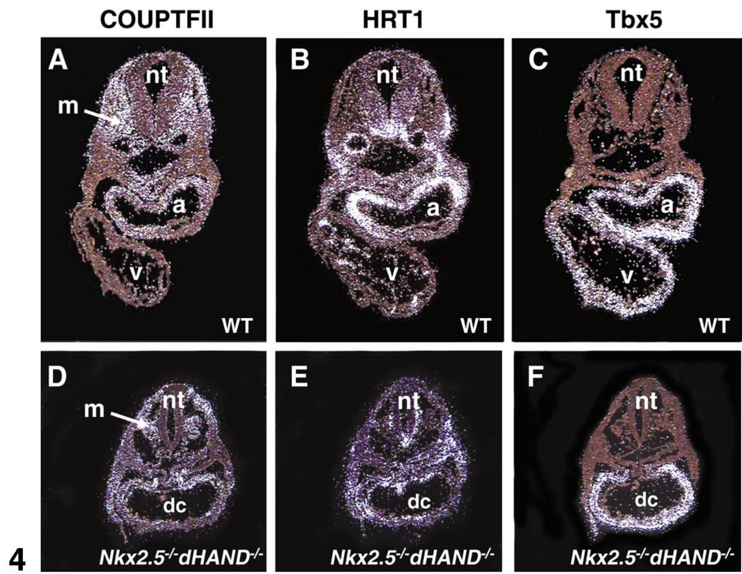

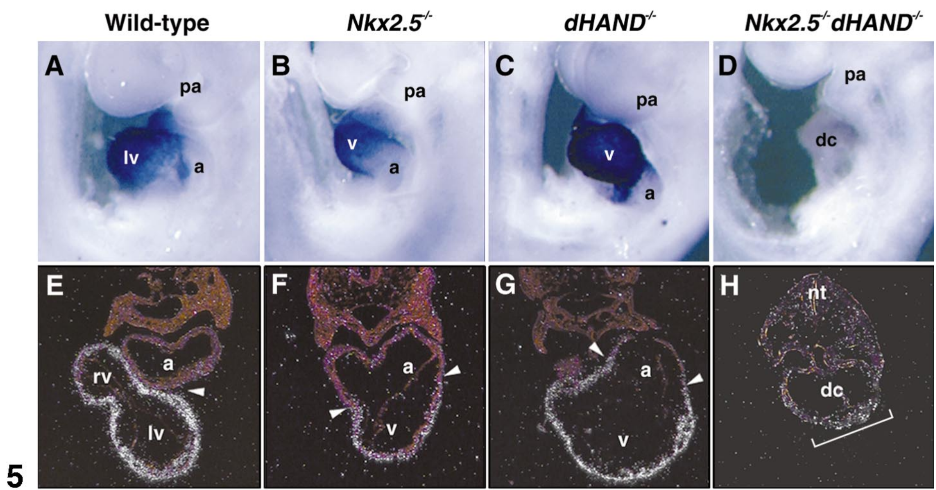

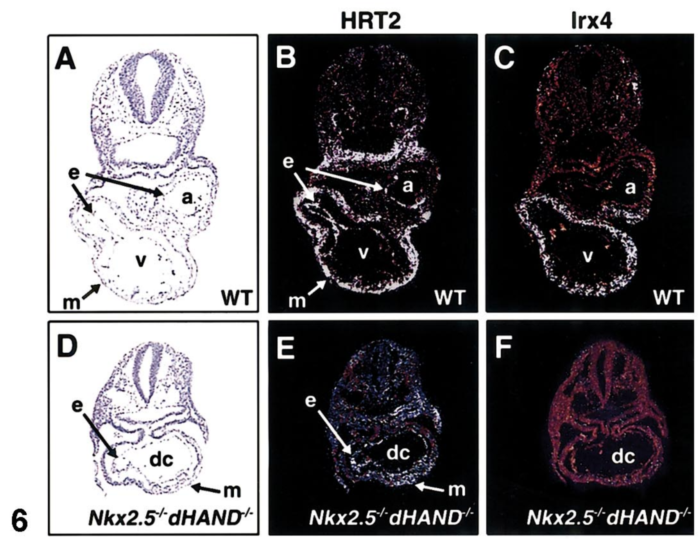

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segment of atrium in *Nkx2.5*$^{-/-}$ mutants. This phenotype was identical to that of complete *Csx/Nkx2.5* mutants recently described (Tanaka *et al.,* 1999). In *dHAND*$^{-/-}$ mutants, the future right ventricular segment was hypoplastic as reported previously (Srivastava *et al.,* 1997), with a single left ventricle and common atrial segment remaining (Figs. 1C, 1G, and 1K). Despite each mutant having what appeared to be a single ventricle and atrial chamber, the morphology of the looped heart tube was distinct in each mutant.

Nkx2.5$^{-/-}$*dHAND*$^{+/-}$ and *Nkx2.5*$^{+/-}$*dHAND*$^{-/-}$ embryos were indistinguishable from *Nkx2.5*$^{-/-}$ or *dHAND*$^{-/-}$ embryos, respectively. However, in *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutants, only one cardiac chamber was apparent at any stage studied (Figs. 1D, 1H, and 1L; Figs. 2D, 2H, and 2L). We will refer to this chamber as the "dorsal chamber" based on its position compared to wild-type atrial and ventricular chambers. The posterior portion of this chamber was connected to bilaterally dilated sinus venosae and the anterior portion was contiguous with a thin outflow tract (Figs. 1L, 2D, and 2L). There was no ventral protrusion of the heart as seen in each single mutant (Figs. 1D and 1H). The single chamber of the *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ heart was slightly oriented to the left (Fig. 1L). Little change in cardiac morphology was noted from E8.25 to E9.25 in the double mutants. By E9.5, double mutant embryos began to develop a pericardial effusion, indicative of cardiac failure, although the heart continued to beat rhythmically. The extraembryonic and embryonic vasculature was disrupted as in each single mutant and this likely also contributed to the embryonic lethality, although the cardiac defect was evident well before the vascular defects.

Histologic analyses confirmed the dorsal and ventral positions of distinct atrial and ventricular chambers, respectively, in *Nkx2.5* mutants (Figs. 2B, 2F, and 2J) and *dHAND* mutants (Figs. 2C, 2G, and 2K). However, in *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ double mutants, only a single dorsal chamber of the heart was apparent at any level of section with no evidence of the ventral chamber observed in each single mutant (Figs. 2D, 2H, and 2L). No formation of trabeculae and little cardiac jelly was observed in the chamber of *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutants. In the most posterior part of the cardiac region, dilated sinus venosae were apparent (Fig. 2L), indicative of hemodynamic insufficiency. Interestingly, an accumulation of cells was consistently observed in a small area in the ventral part of the single chamber that is described in further detail below (Fig. 2H, bracket).

Specification of Atrial Cardiomyocytes in Nkx2.5$^{-/-}$*dHAND*$^{-/-}$ *Hearts*

To determine whether the single cardiac chamber in *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutants had differentiated and adopted a particular chamber fate, we performed a detailed molecular analysis of the double mutant hearts. MLC-1A and -2A are some of the earliest markers of cardiomyocyte differentiation and are expressed in both atrium and ventricle during early heart development (Lyons *et al.,* 1990; Kubalak *et al.,* 1994). Both MLC1A (data not shown) and MLC2A (Figs. 3A–3D) were expressed at normal levels in each single mutant and in *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ hearts between E8.25 and E9.25.

In *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutants, a ventral chamber was not found and the prominent chamber resembled an atrium morphologically. We therefore examined a series of markers by *in situ* hybridization to determine whether the dorsal chamber in *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutants was indeed an atrium. COUP-TFII is a member of the steroid/thyroid hormone receptor superfamily that is expressed in mesenchymal cells throughout the embryo during organogenesis, and specifically in the atria of the developing heart (Pereira *et al.,* 1995). COUP-TFII was expressed at normal levels in the dorsal chamber of either *Nkx2.5*$^{-/-}$ or *dHAND*$^{-/-}$ mu-

FIG. 4. Atrial markers are expressed in *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutant hearts. Serial transverse sections of E9.25 wild-type (WT) (A–C) and *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ (D–F) embryos were examined for cardiac expression of the atrial markers, COUP-TFII (A, D), HRT1 (B, E), and Tbx5 (C, F) by ^{35}S section *in situ* hybridization. COUP-TFII, a mesenchymal (m) and atrial (a) marker, was expressed in WT (A) and *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ (D) embryos. HRT1 was also expressed in the atria of WT (B) and the single dorsal chamber (dc) in *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ (E) embryos. Tbx5 was expressed in the atria and left ventricle (v) of WT hearts (C) and in the dc of *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutants (F). nt, neural tube.

FIG. 5. Ventricular dysgenesis in *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ embryos. MLC2V, a ventricular marker, was expressed in wild-type (A), *Nkx2.5*$^{-/-}$ (B), and *dHAND*$^{-/-}$ (C) embryos at E9.25, but not in *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ (D) embryos by whole-mount *in situ* hybridization. (A–D) Left lateral views focusing on the cardiac region. ^{35}S section *in situ* hybridization of MLC2V on transverse sections through E9.25 embryos revealed distinct atrial (a) and ventricular (v) chambers in wild-type (E), *Nkx2.5*$^{-/-}$ (F), and *dHAND*$^{-/-}$(G) embryos. Arrowheads demarcate the atrio-ventricular junction. In E9.25 *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ embryos, MLC2V was expressed in only a small number of cardiomyocytes, along the ventral surface (H, bracket). pa, pharyngeal arch; lv, left ventricle; nt, neural tube; rv, right ventricle; dc, dorsal chamber.

FIG. 6. Irx4 expression is abolished in *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutant hearts. Serial transverse sections of E9.25 wild-type (WT) (A–C) and *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ (D–F) embryos were examined for cardiac expession of the ventricular transcription factors, HRT2 (B, E) and Irx4 (C, F) by ^{35}S section *in situ* hybridization. Bright field images of sections are shown in (A) and (D). In WT, HRT2 was expressed in the endocardium (e) and myocardium (m) of the ventricle (v) and in the endocardium of the atrium (a) (B). In the double mutant (E), HRT2 was expressed in the endocardium and in a few myocardial cells along the ventral surface (brackets) of the single dorsal chamber (dc). In contrast, Irx4 was expressed in the ventricular myocardium of wild-type embryos (C) but was undetectable in the heart of *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutants, even in the MLC2V- and HRT2-expressing cells (brackets) (F).

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tants (data not shown), suggesting that these chambers had atrial characteristics and that regulation of *COUP-TFII* was independent of both Nkx2.5 and dHAND. Similarly, in *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutants, COUP-TFII was expressed in the single dorsal chamber, suggesting that this chamber may be composed of cells specified to be atrial in nature (Figs. 4A and 4D). COUP-TFII was also expressed in mesenchymal cells throughout the embryo. HRT1 is a member of a recently described subclass of bHLH transcription factors which is expressed in the atria but not ventricles during murine cardiac development (Nakagawa *et al.,* 1999). Similar to COUP-TFII, HRT1 was expressed in the dorsal chamber of both *Nkx2.5*$^{-/-}$ and *dHAND*$^{-/-}$ mutants (data not shown), and in the chamber of *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutants (Figs. 4B and 4E). Finally, expression of Tbx5, a marker of atrial and left ventricular precursors (Bruneau *et al.,* 1999; Liberatore *et al.,* 2000), was unaffected in *Nkx2.5* and *dHAND* single (data not shown) or double mutants (Figs. 4C and 4F). Taken together, these data suggest that cardiomyocyte differentiation and atrial specification occurred in *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutants and that the single dorsal cardiac chamber of *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ embryos was molecularly specified as an atrium.

Ventricular Dysgenesis in Nkx2.5$^{-/-}$*dHAND*$^{-/-}$ *Hearts*

To determine whether any ventricular formation or differentiation had occurred, we examined the expression of a ventricular-specific marker, MLC2V, in *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ embryos. MLC2V is one of the earliest markers for ventricular development and is expressed exclusively in the ventricles and not in the atrium by E8.5 in wild-type embryos (O'Brien *et al.,* 1993). Previous reports showed that MLC2V was downregulated in *Nkx2.5*-null hearts and was detectable only in a small region of the heart at E8.5–E9.0 (Lyons *et al.,* 1995). At E9.25, we were able to detect low levels of MLC2V expression in the ventral chamber but not in the dorsal, putative atrial, chamber of *Nkx2.5*$^{-/-}$ mutant hearts, similar to the recently described *Csx/Nkx2.5* mutant (Tanaka *et al.,* 1999). A sharp border of expression was evident between the ventral and dorsal regions of the looped heart tube (Figs. 5B and 5F), suggesting that two distinct chambers were present in *Nkx2.5* mutants. In *dHAND*$^{-/-}$ embryos, MLC2V was exclusively expressed in the ventricle at levels similar to wild-type embryos, but was not detected in the atrial chamber (Figs. 5C and 5G). In sharp contrast, MLC2V was difficult to detect in *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ hearts by whole-mount *in situ* hybridization at any stage, suggesting severe ventricular hypoplasia (Fig. 5D). In *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutants, MLC2V transcripts were detectable by section *in situ* hybridization only in the ventral region of the heart tube where an accumulation of cells was observed (Fig. 5H). This result indicated that a small number of cardiomyocytes could express ventricular-specific markers in the absence of Nkx2.5 and dHAND and that these cells had accumulated along the ventral surface of the single cardiac chamber. However, MLC2V was not detectable throughout the remainder of the double mutant heart, consistent with the absence of a ventricular chamber.

Irx4 Expression Is Abolished in Nkx2.5$^{-/-}$*dHAND*$^{-/-}$ *Mutant Hearts*

To further investigate the molecular basis for ventricular dysgenesis in *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutants, we used a candidate gene approach to search for ventricular-specific transcription factors that might be dysregulated in the double mutants. The bHLH protein, HRT2, is normally expressed in the myocardial layer of the ventricles and in the endocardial layers of the atria and ventricles (Nakagawa *et al.,* 1999). By section *in situ* hybridization analysis, HRT2 transcripts were detectable in the endocardium of the presumptive atrial chamber of *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutants, but were not present in most of the myocardium of this chamber. Interestingly, HRT2 transcripts were present in the ventral portion of *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ hearts where the unusual collection of MLC2V-expressing cells was observed (Figs. 6B and 6E). These observations were consistent with the notion that specification of a subset of ventricular cells had occurred in the *Nkx2.5*$^{-/-}$-*dHAND*$^{-/-}$ mutant, although the number of ventricular cells was far fewer than normal and ventricular morphogenesis did not occur. To search further for mediators of ventricular dysgenesis, the expression of *Irx4*, a ventricular-specific homeobox gene, was examined. Irx4 has been shown to be downregulated in *Nkx2.5* or *dHAND* single mutants, although transcripts are still detectable in the single mutants (Bruneau *et al.,* 2000). However, in *Nkx2.5*$^{-/-}$-*dHAND*$^{-/-}$ mutants, Irx4 expression was completely abolished, even in the ventrally located HRT2- and MLC2V-positive cells that would have been expected to express Irx4 (Figs. 6C and 6F).

eHAND Expression Is Absent in Nkx2.5 Mutant Precardiac Mesoderm

While eHAND is downregulated in E9.5 *Nkx2.5* mutants, it has not been clear whether this is a specific effect on eHAND regulation or if the observation may reflect the absence of a left ventricle in *Nkx2.5* mutants. To determine whether *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutants are effectively null for both dHAND and eHAND prior to ventricular formation, we examined the expression of eHAND in *Nkx2.5* mutants at E7.75, just as the precardiac mesoderm becomes arranged into the cardiac crescent. Even at this early stage, eHAND transcripts were undetectable in *Nkx2.5* mutants in the precardiac mesoderm, although robust expression was maintained in the remainder of the lateral mesoderm bilaterally (Figs. 7A and 7D). Regulation of eHAND was studied further by crossing mice harboring a *lacZ* marker in the *eHAND* locus (Firulli *et al.,* 1998) into the *Nkx2.5*-null genetic background. In this manner, we were able to demonstrate the absence of *lacZ* expression in the left ventricular precursors of *Nkx2.5* mutants at E8.0–E8.5 (Figs. 7B

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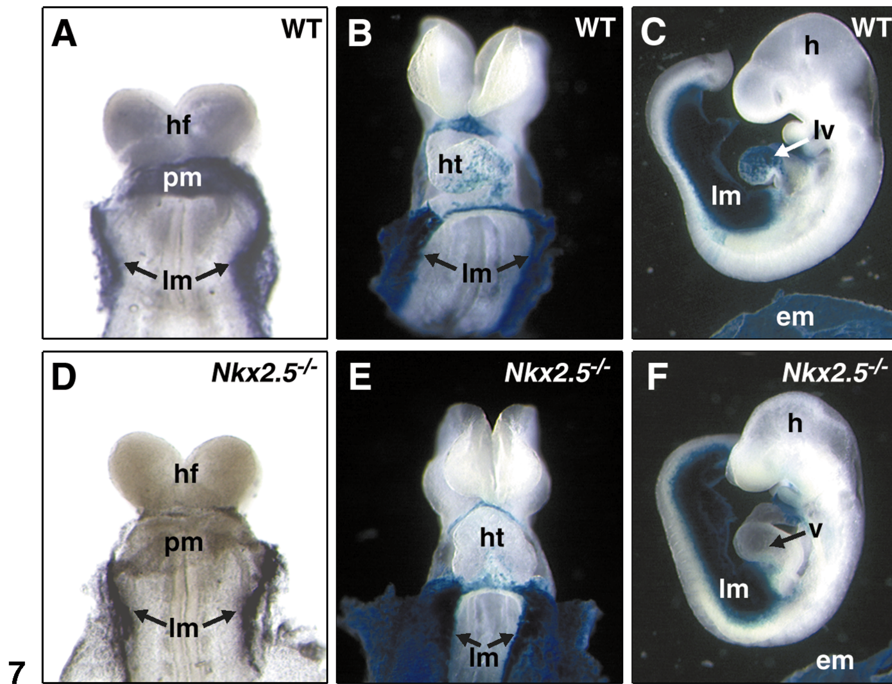

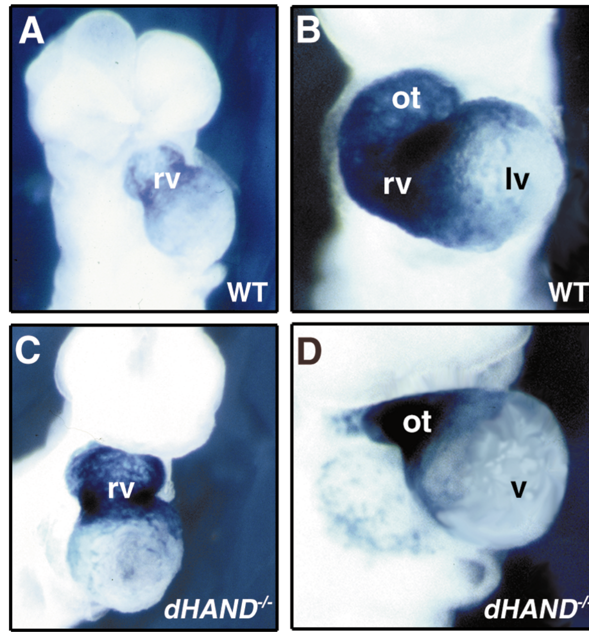

FIG. 7. eHAND downregulation in *Nkx2.5*$^{-/-}$ precardiac mesoderm and early heart tube. Frontal view of whole-mount *in situ* hybridization on E7.75 wild-type (WT) and *Nkx2.5*$^{-/-}$ embryos revealed absence of eHAND expression in the precardiac mesoderm (pm) of *Nkx2.5* mutants (A, D). Expression of the *eHAND*^{lacZ} allele demonstrated *lacZ*-positive cells in the future left ventricle segment of the heart tube (ht) of WT (B) but not *Nkx2.5*$^{-/-}$ (E) E8.0–E8.5 embryos in frontal views. *eHAND*^{lacZ} was expressed symmetrically in the right and left lateral mesoderm (lm) of WT and mutant embryos. In E9.5 embryos, *eHAND*^{lacZ} was expressed in the left ventricle (lv) and lm of WT embryos but not in the ventricle (v) of *Nkx2.5* mutants, as seen in left lateral views. h, head; hf, head fold.

FIG. 8. Failure of ventricular expansion in *dHAND*$^{-/-}$ hearts. Expression of *lacZ* under control of the right ventricular-specific *MLC2V* promoter in wild-type (WT) (A, B) and *dHAND* $^{-/-}$ (C, D) embryos at E8.25 (A, C) and E9.0 (B, D) is shown in frontal views of the heart. ot, outflow tract; v, ventricle; lv, future left ventricle; rv, future right ventricle.

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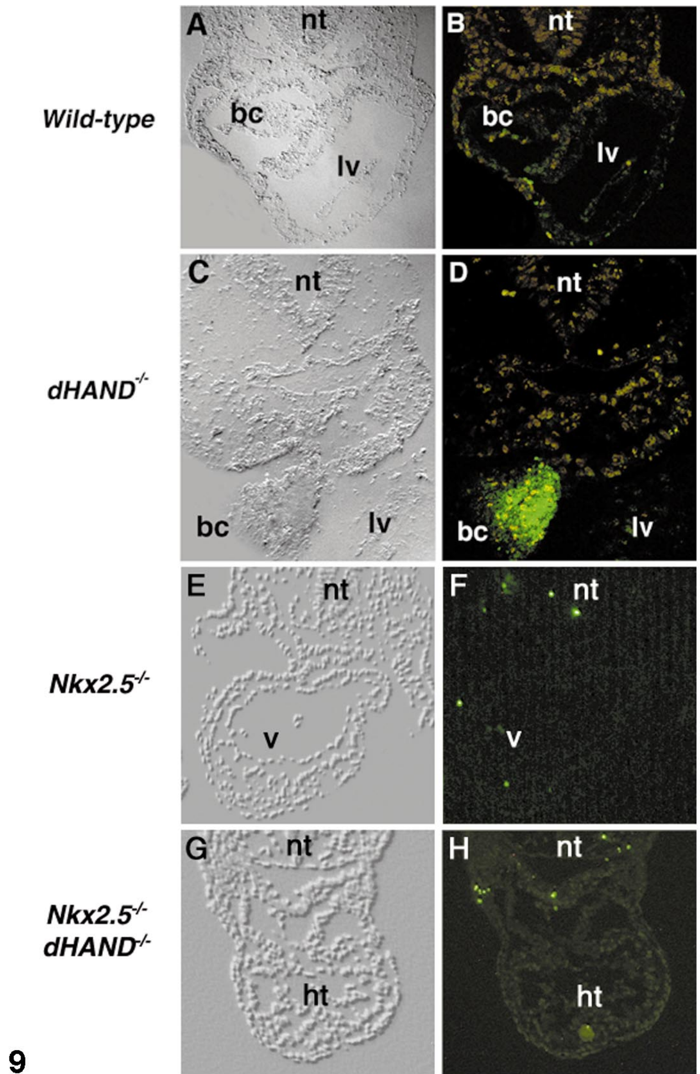

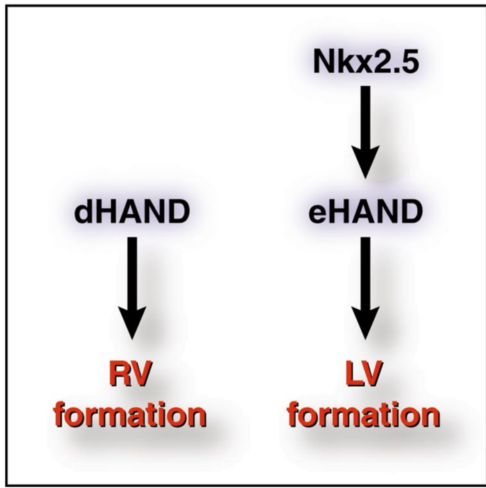

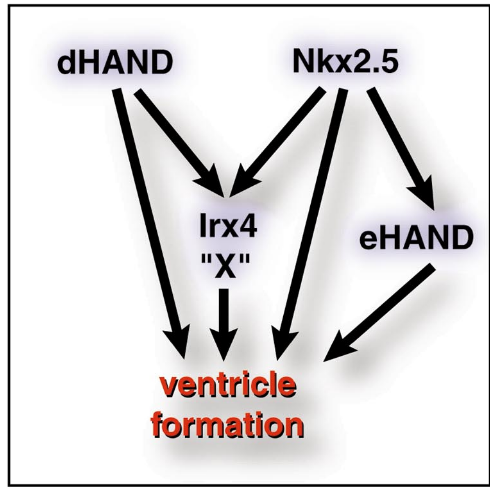

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and 7E). It is worth noting that no asymmetry of *lacZ* expression was noted along the left–right axis in the sinus venosae or lateral mesoderm (Figs. 7B and 7E). Finally, downregulation of *eHAND* in E9.25 *Nkx2.5* mutants was confirmed by the absence of *lacZ* expression in the ventricle of mutants (Figs. 7C and 7F). Thus, mice lacking *Nkx2.5* and *dHAND* appear to be effectively null for both dHAND and eHAND prior to cardiac formation.

Ventricular Expansion and Cell Death Defects in Mutant Hearts

Conceptually, cardiac ventricular formation can be divided into two phases: (1) the initial specification of ventricular cell lineages, and (2) subsequent expansion of the ventricular segment through a combination of cell movement and proliferation at the looping stage. The accumulation of ventricular cells along the ventral surface of the atrial chamber in *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutants suggests that some cardiomyocytes are specified as ventricular cardiomyocytes, but those ventricular cells may fail to increase in number to form a full chamber. Because eHAND is not expressed in the right ventricle, the right ventricular precursors of *dHAND*-null embryos are also effectively null for both HAND proteins. We therefore used the *dHAND*-null model to test the hypothesis that the HAND proteins are required for ventricular expansion. To accomplish this, we traced the fate of right ventricular cells in the *dHAND*-null background using mice harboring *lacZ* under control of the right ventricular-specific promoter of *MLC2V* (Ross *et al.,* 1996). By crossing the *MLC2V-lacZ* transgene into the *dHAND*-null background, we found that right ventricular cells were indeed specified at the straight heart tube stage in the absence of dHAND (Figs. 8A and 8C). However, during cardiac looping when the ventricular chambers are thought to expand from the ventral surface of the heart tube, the right ventricular segment, as marked by *lacZ*-expressing cells, failed to expand (Figs. 8B and 8D).

To determine whether the mechanism of ventricular hypoplasia was associated with impairments of cell proliferation or survival, transverse sections of E8.25 and E8.5 hearts were concurrently examined for alterations in levels of apoptosis and cell proliferation. The right ventricular precursors in the bulbus cordis of mice homozygous null for *dHAND* displayed a tremendous amount of apoptosis as determined by TUNEL assay (Fig. 9D). Consistent with previous observations (Tanaka *et al.,* 1999), no changes in programmed cell death or cell proliferation were observed in wild-type or *Nkx2.5*$^{-/-}$ mutants (Figs. 9B and 9F). Interestingly, *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ E8.25 hearts showed minimal evidence of apoptosis on the ventral surface (Fig. 9H), suggesting the early absence of ventricular cells or early loss of ventricular cells in the absence of both genes. Electron microscopy of tissue sections confirmed evidence of apoptosis in *dHAND* mutant embryos. Cell proliferation was observed at near normal levels in all mutants as determined by BrdU incorporation (data not shown). These results indicate that inappropriate apoptosis of specified ventricular cells results in failure of ventricular expansion in *dHAND*, but not *Nkx2.5*, single mutants.

DISCUSSION

Unlike in skeletal muscle, neuronal, or hematopoietic development, identification of a single transcription factor responsible for cardiogenesis in vertebrates has been elusive. Instead, a search for the aggregate factors and molecular pathways involved in ventricular and atrial development may represent a more obtainable goal. Here, we investigated the combinatorial activities of two critical transcription factors, Nkx2.5 and dHAND, involved in cardiac development. Early cardiac markers suggest that cardiomyocyte specification and differentiation can occur even in the combined absence of Nkx2.5 and dHAND. Morphologic and molecular analyses indicated that the hearts of *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ embryos became patterned into an atrium but that morphogenesis of the right and left ventricles failed to occur and embryos died between E9.5 and 10.5. A small pool of cardiomyocytes expressing ventricular-specific markers was detectable in the ventral portion of the atrial chamber but failed to form the ventricular chambers. These results provide the first demonstration of gene mutations that result in ablation of the entire ventricular segment of the mammalian heart.

Similarity of Nkx2.5$^{-/-}$dHAND$^{-/-}$ and hands off Mutants

The *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$phenotype is reminiscent of the zebrafish mutant, *hands off*, that is caused by a mutation in the zebrafish *dHAND* gene (Yelon *et al.,* 2000). Zebrafish have only a single ventricle and a single *HAND* gene identified to date that has closest homology to *dHAND* (Angelo *et al.,* 2000). In the *hands off* mutant, cardiomyocyte specification occurs, but myocytes are primarily atrial

FIG. 9. Programmed cell death in mutant hearts. Transverse sections of E8.25 wild-type and mutant hearts are shown in bright field images (A, C, E, F) and after TUNEL assay (B, D, F, H). Bright green cells represent TUNEL-positive cells undergoing apoptosis. nt, neural tube; lv, left ventricle; v, ventricle; bc, bulbus cordis (future right ventricle).

FIG. 10. Model for transcriptional regulation of ventricular formation. Model 1, dHAND and Nkx2.5 (via eHAND) regulate right ventricle (RV) and left ventricle (LV) formation, respectively. Model 2, dHAND and Nkx2.5, in addition to their independent regulation of some pathways, together regulate Irx4 and yet unknown ventricular-specific factors "X" during ventricular formation.

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and ventricular formation does not occur. An irregular distribution of ventricular myosin heavy chain in a few myocardial cells was observed. Thus, the *hands off* phenotype resembled the *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutant in that ventricular specification occurred in some cardiomyocytes, but these cells failed to subsequently form a ventricular chamber.

In spite of these similarities, the *hands off* phenotype is more severe than the *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ phenotype. Morphologically, *hands off* mutants develop two small lateral clusters of myocardial cells that never fuse together in the midline, unlike *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutants that form a midline heart tube. This discrepancy may be explained by the residual eHAND expression in the lateral plate mesoderm (lm) of *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutants that might compensate for the absence of dHAND in the lm. In addition to the morphologic differences, myocardial cells in *hands off* mutants fail to maintain *Tbx5* expression. This is in contrast to the *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutants that demonstrate robust *Tbx5* expression in the remaining atrial chamber, suggesting differences in genetic regulation of *Tbx5* in mice and zebrafish.

Potential Molecular Mechanisms for Ventricular Dysgenesis

Although the phenotype of *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ embryos is unambiguous, there are several potential mechanistic interpretations. Because our studies indicate that eHAND is downregulated in the precardiac mesoderm of *Nkx2.5* mutants, it is attractive to hypothesize that the phenotype of *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ mutants reflects the loss of both dHAND and eHAND expression. Similarity of the *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ phenotype in the mouse and *hands off* phenotype in the zebrafish described above may support this hypothesis. Since dHAND and eHAND are expessed in a somewhat complementary fashion in the right and left ventricles in mice, it is possible that dHAND and eHAND may have redundant roles and are required for expansion of the right and left ventricular segment, respectively, at the looping stage (Fig. 10, model 1). While genetic redundancy has not been established in the mouse heart, dHAND's role in anterior–posterior patterning of the limb bud (Fernandez-Teran *et al.,* 2000; Charite *et al.,* 2000) can be functionally substituted by eHAND in the limb (D. McFadden, D.S., and E.N.O., unpublished observations). Generation of a *dHAND* and *eHAND* double-null mouse using a conditional *eHAND* deletion is in progress and will determine the extent to which eHAND downregulation contributes to the *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ phenotype.

While a simple model as described above is attractive, it is unlikely that the role of Nkx2.5 in cardiac development is solely based on its regulation of *eHAND* expression. Rather, it is likely that Nkx2.5 regulates genes that are independent of pathways regulated by the HAND proteins. However, the apparent genetic interaction of Nkx2.5 and dHAND-related pathways might indicate that Nkx2.5 and dHAND also synergistically regulate a set of factors important for ventricular development. While *HRT2* is not regulated by either pathway, the ventricular homeobox gene, *Irx4*, is an example of a gene dependent on both Nkx2.5 and dHAND (Fig. 10, model 2). The complete lack of Irx4 expression in the ventricular-specific cells of *Nkx2.5*$^{-/-}$-*dHAND*$^{-/-}$ mutants is more severe than the partial downregulation observed in either single mutant. Thus, it is possible that the ventricular dysgenesis also reflects the absence of Irx4 and other, yet unknown, ventricular-specific factors, that are normally regulated by Nkx2.5 and dHAND in a cooperative fashion. Determining whether Nkx2.5 and dHAND synergistically activate *Irx4* will await analysis of the *Irx4* regulatory region.

Ventral Expansion of Ventricular Cardiomyocytes

Concurrent with the segmental model of cardiac development, a model has been proposed in which growth of the ventral aspect of the linear heart tube gives rise to the outer curvature of the looped heart and results in ventricular expansion (de Jong *et al.,* 1997; Christoffels *et al.,* 2000). The expression patterns of dHAND and eHAND are consistent with this model, with expression of eHAND on the ventral surface of the straight heart tube but not on the dorsal side (Thomas *et al.,* 1998a; Biben and Harvey, 1997). The ventricular remnant cells on the ventral surface of *Nkx2.5*$^{-/-}$-*dHAND*$^{-/-}$ hearts are a subtle but important finding. The presence of these cells, which express ventricular markers, supports the model of ballooning growth of the ventricular chamber from the ventral surface of the heart tube. In the absence of appropriate signals from the Nkx2.5 and HAND-related pathways, the ventrally located ventricular cells may fail to expand into a ventricular segment, remaining in a more primitive state in which they occupy only the ventral surface of the heart tube. While the "ballooning" model of cardiogenesis has been inferred by cell fate analyses and gene expression domains, this observation represents some of the more convincing functional evidence supporting this model.

There are several potential mechanistic explanations for the failure of expansion of ventrally located ventricular cells in *Nkx2.5*$^{-/-}$*dHAND*$^{-/-}$ embryos. While our results suggest that ventricular specification may occur in at least some cells, it is possible that other preventricular cells are respecified into other cardiac lineages in the absence of Nkx2.5 and dHAND. In addition, it is worth considering that the few cells expressing ventricular markers may be doing so aberrantly and thus may not represent ventricular cells. Alternatively, the defect may lie in regulation of the number of ventricular cells available to contribute to a ventricular chamber. Although our studies indicate that cells continue to proliferate to some degree in the absence of dHAND and Nkx2.5, a marked dysregulation of apoptosis was observed. Programmed cell death had been examined previously (Tanaka *et al.,* 1999) and, consistent with our observations, was not found to play a major role in the cardiac defects described in *Nkx2.5* mutants. In contrast,

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apoptosis does play a role in pharyngeal arch hypoplasia in *dHAND* mutants (Thomas *et al.,* 1998b), and here we show that a similar mechanism is involved during ventricular hypoplasia, suggesting that dHAND may function to promote survival of cells during their period of rapid growth. The absence of apoptotic cells in *Nkx2.5* mutants may reflect distinct functional roles of Nkx2.5 and dHAND; alternatively, the continued expression of dHAND in the ventricles of *Nkx2.5* mutants may be sufficient to overcome any survival defects that might have resulted from downregulation of eHAND. The paucity of ventricular cells and the absence of apoptotic cells at E8.25 in *Nkx2.5*$^{-/-}$-*dHAND*$^{-/-}$ embryos may reflect an earlier loss of ventricular cardiomyocytes, possibly at the cardiac crescent stage, or altered specification of ventricular cells.

Dissection of the complex molecular pathways involved in ventricular specification, differentiation and growth will likely provide the basis for understanding the pathogenesis of hypoplastic ventricle syndromes. In addition, deciphering the molecular code for expansion of a population of cardiomyocytes will be increasingly important as efforts to generate cardiomyocytes from endogenous cardiac or noncardiac cells progress. The pathways explored here provide evidence for a molecular network that is essential for cardiomyocyte expansion and chamber formation that may be exploited in the future.

ACKNOWLEDGMENTS

We thank R. S. Ross and K. R. Chien for *MLC2V-lacZ* transgenic mice, C. E. Seidman and B. G. Bruneau for Tbx5 and Irx4 probes, and S. Y. Tsai for COUP-TFII probe; S. Johnson for manuscript and figure preparation; T. Justus for technical assistance; and members of the Srivastava lab for helpful discussions. We also thank members of the Molecular Pathology Core (C. Pomajzl, J. Shelton, and J. Richardson) for assistance with section *in situ* hybridizations. This work was supported by grants from the NHLBI/NIH (1-P50-HL61033-01, 1-R01-HL62591-01, 5-R01-HL57181-04), March of Dimes Birth Defects Foundation and Smile Train, Inc. (to D.S.). O.N. was supported by the Department of Cardiovascular Medicine, Kumamoto University School of Medicine, Japan Heart Foundation & Bayer Yakuhin Research Grant Abroad, Uehara Memorial Foundation and Yamanouchi Foundation for Research on Metabolic Disorders. E.N.O. was supported by grants from the NIH/NHLBI, the D. W. Reynolds Center for Clinical Cardiovascular Research and the McGowan Charitable Trust.

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Received for publication July 5, 2001
Revised August 2, 2001
Accepted August 2, 2001
Published online October 11, 2001

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