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GATA4 is a dosage-sensitive regulator of cardiac morphogenesis

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Abstract

Normal heart development is orchestrated by a set of highly conserved transcription factors that includes GATA4, Nkx2-5, and Tbx5. Heterozygous mutation of each of these genes causes congenital heart disease in humans. In mouse models, haploinsufficiency for Nkx2-5 or Tbx5 resulted in an increased incidence of structural heart disease, confirming that normal heart development is sensitive to small changes in expression levels of Nkx2-5 and Tbx5. However, mice haploinsufficient for GATA4 have not been reported to have cardiac abnormalities. We generated two new GATA4 alleles, *GATA4*^{H} and *GATA4*^{flox}. GATA4$^{flox/flox}$ embryos expressed 50% less GATA4 protein in the heart and survived normally. In contrast, GATA4$^{H/H}$ embryos expressed 70% less GATA4 protein in the heart and died between days 13.5 and 16.5 of gestation. These embryos had common atrioventricular canal (CAVC), double outlet right ventricle (DORV), hypoplastic ventricular myocardium, and normal coronary vasculature. Myocardial hypoplasia was associated with diminished cardiomyocyte proliferation. Hemodynamic measurements demonstrated that these embryos had normal systolic function, severe diastolic dysfunction, and atrioventricular regurgitation. Surprisingly, expression levels of the putative GATA4 target genes *ANF*, *BNP*, *MEF2C*, *Nkx2-5*, *cyclin D2*, and *BMP4* were unchanged in mutant hearts, suggesting that GATA4 is not a dose-limiting regulator of the expression of these genes during later stages of embryonic cardiac development. These data demonstrate that multiple aspects of embryonic cardiac morphogenesis and function are exquisitely sensitive to small changes in GATA4 expression levels.

Keywords: GATA4; Cardiac morphogenesis; Heart

Introduction

Congenital heart disease is the most common major congenital anomaly, with an incidence of 1 in 200 live births (Hoffman, 1995). Growth and maturation of the fetal heart is regulated by a set of highly conserved transcription factors that includes GATA4, Nkx2-5, and Tbx5 (Srivastava and Olson, 2000). Mutation of one copy of these genes is associated with cardiac malformations in humans, presumably as a result of haploinsufficiency (Basson et al., 1997; Garg et al., 2003; Pehlivan et al., 1999; Schott et al., 1998). The sensitivity of heart development to dosage of Nkx2-5 or Tbx5 was recapitulated in mouse models in which targeted ablation of one copy of these genes resulted in an increased frequency of cardiac malformations (Biben et al., 2000; Bruneau et al., 2001). However, the pathogenesis of heart malformations in humans with missense or nonsense mutations in GATA4 is less well understood since haploinsufficiency for GATA4 in mice was not reported to be associated with cardiac malformations (Kuo et al., 1997; Molkentin et al., 1997).

The zinc finger transcription factor GATA4 and its binding partner FOG2 are essential components of the fetal

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cardiac transcriptional program. Null mutation of GATA4 resulted in abnormal ventral folding of the embryo, failure to form a single ventral heart tube, and lethality by embryonic day (E) 10.5 (Kuo et al., 1997; Molkentin et al., 1997). GATA4 is also essential for later cardiac morphogenesis since embryos homozygous for a GATA4 point mutation (*GATA4*^{Ki}) that abolished interaction with FOG2 died just after E12.5. These embryos had cardiac malformations that included hypoplasia of the compact myocardium, common atrioventricular canal (CAVC), and double outlet right ventricle (DORV) (Crispino et al., 2001). Similarly, FOG2 null embryos had hypoplasia of the compact myocardium, CAVC, and normally related great arteries (Tevosian et al., 2000). Strikingly, both GATA4$^{Ki/Ki}$ and FOG2 null hearts were reported to have an absence of the coronary vasculature (Crispino et al., 2001; Tevosian et al., 2000).

Using gene targeting, we generated a new hypomorphic allele of GATA4, *GATA4*^{H}, which expressed reduced amounts of GATA4 protein. In this report, we characterize mouse embryos homozygous for this hypomorphic allele and demonstrate that the level of cardiac GATA4 expression is an important regulator of cardiomyocyte proliferation, cardiac morphogenesis, and embryo survival.

Materials and methods

Gene targeting

A bacterial artificial chromosome containing GATA4 genomic DNA was isolated from a 129/SvJ BAC library (Genome Systems). A targeting vector was constructed using the following fragments (Fig. 1a): (1) a thymidine kinase gene; (2) a 1268-bp *Nsi*I–*Nhe*I fragment containing GATA4 genomic DNA including the 5′ end of exon 2; (3) an oligonucleotide containing a loxP site and an *Xho*I site; (4) a 1302-bp *Nhe*I–*Eco*RV fragment containing GATA4 genomic DNA including the 3′ end of exon 2; (5) an Frt-Kan-Neo-Frt-loxP cassette; and (6) a 2762-bp *Eco*RV–*Eco*RI fragment containing GATA4 genomic DNA from the second intron. The targeting vector was used to modify the GATA4 locus of embryonic stem cells by homologous recombination. Neomycin-resistant clones were tested for correct gene targeting by Southern blotting using 5′ and 3′ probes (Fig. 1b) and by PCR using primer pairs spanning the targeting vector and flanking genomic DNA (not shown). To confirm proper activity of loxP and Frt sites, colonies derived from transient transfection of a targeted ES cell clone with a plasmid expressing either Cre or Flp recombinase were tested for loss of Neo resistance. Seven out of 48 colonies were sensitive to G418 after transient Flp expression. Twenty-three out of 48 colonies were sensitive to G418 after transient Cre expression. No colonies were sensitive to G418 after transfection with a control plasmid. Southern blotting of

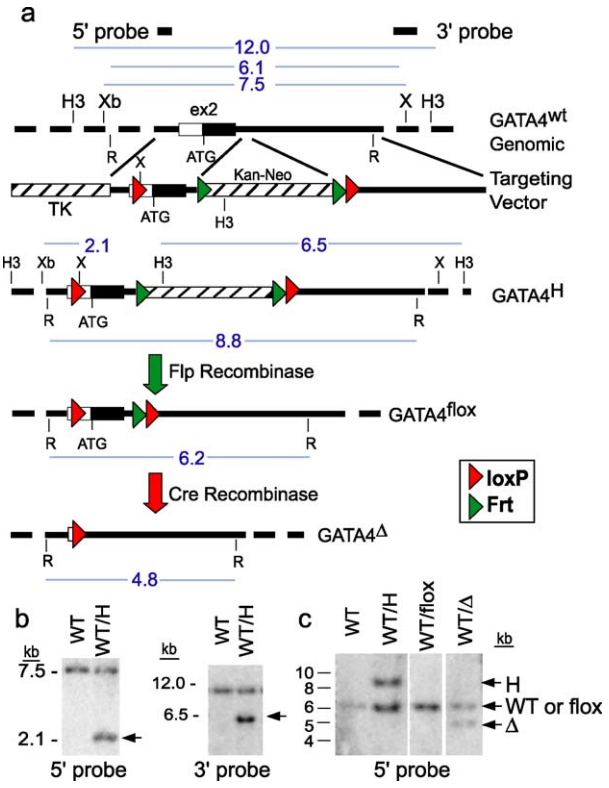

Fig. 1. GATA4 alleles produced by gene targeting. (a) Modification of targeted locus by expression of Cre and Flp recombinases. Open box, 5′ UTR; black box, coding region; solid line, regions of homology between genomic locus and targeting vector; dashed line, flanking genomic DNA. Triangles, loxP and Frt sequences; H3, *Hin*dIII; R, *Eco*RI; Xb, *Xba*I; X, *Xho*I; TK, thymidine kinase; Kan-Neo, Kan resistance and pgk-Neo cassettes. Blue lines indicated expected length of diagnostic fragments used in Southern blotting. (b) Proper homologous recombination verified by Southern blotting. ES cell genomic DNA was digested with *Hin*dIII (3′ probe) or *Xba*I and *Xho*I (5′ probe). Arrows indicate bands with sizes expected following proper targeting. (c) Proper recombination of Frt and loxP sites after transient expression of Flp (WT/flox lane) or Cre (WT/Δ lane) recombinase in a properly targeted ES cell clone. ES cell genomic DNA was digested with *Eco*RI and hybridized to the 5′ probe.

Neo-sensitive colonies confirmed expected DNA recombination (Fig. 1c).

Mice

Chimeric mice were generated from one properly targeted clone by blastocyst injection. Germline transmission was achieved by mating to C57BL6 mice. Mice used in this study were on a mixed 129/C57BL6 genetic background, backcrossed to C57BL6 2–5 generations. DNA extracted from tail biopsies and yolk sacs were genotyped by PCR (primers and protocols available on request). Susan Dymecki provided hactB::FLPe transgenic mice (Rodriguez et al., 2000). EIIaCre mice were obtained from Fred Alt (Williams-Simons and Westphal, 1999). Timed matings were performed by examining females for vaginal plugs, with noon of the day of the

plug defined as day 0.5. For BrdU labeling, 2 mg BrdU was administered by intraperitoneal injection 2 h before sacrifice.

Embryo physiology

Preparation and hemodynamic evaluation of mouse embryos were performed as described previously (Ishiwata et al., 2003). In brief, each embryo was exposed in a bath containing Hank's balanced salt solution at 37°C. The ventricular systolic and diastolic areas were obtained by tracing the epicardial border on frontal images recorded with a color CCD camera mounted on a stereoscopic microscope. Blood velocities across ventricular inflow were measured with a 20-MHz pulsed Doppler velocimeter. Blood pressure in ventricles was measured with a servo-null micropressure system attached to a 10-μm glass pipette that was inserted directly into the ventricle.

Histology

Embryos were fixed overnight in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Five- to 10-μm sections were cut and stained with hematoxylin and eosin. Three-dimensional reconstruction was performed using SURFdriver software. BrdU staining was performed on paraffin sections using BrdU antibody (Sigma), the M.O.M. staining kit (Vector), and tyramide-Cy3 (Perkin-Elmer) as the HRP substrate. Nuclei were counterstained with TOPRO3 (Molecular Probes), and cardiomyocytes were stained with a rabbit desmin antibody (BioMeda) and donkey anti-rabbit Alex488 (Molecular Probes). TUNEL staining was performed on paraffin sections using the TMR Red in situ Death Detection Kit (Roche). Fluorescent images were acquired with a confocal microscope (Bio-Rad).

Gene expression

In situ hybridization was performed as described previously (Tanaka et al., 1999), using ^{35}S-labeled probes for GATA4 (Molkentin et al., 1997), ANF (Zeller et al., 1987), and N-myc (Tanaka et al., 1999). Quantitative real-time RT-PCR (qRT-PCR) was performed on an ABI7700 using Taqman probes for *GATA4*, *GATA6*, *MEF2C*, *Nkx2-5*, *BMP4*, *cyclins D1-D3*, and *HOP* and normalized to 18 S rRNA. *ANF*, *BNP*, and *GAPDH* were measured by qRT-PCR using Sybr Green detection. Primer and probe sequences are available at http://www.cardiogenomics.org. Total RNA from E12.5 or E13.5 hearts was isolated using the RNeasy kit (Qiagen) with on-column DNase digestion. Each sample consisted of two to three pooled hearts, assayed in duplicate. Three samples were analyzed per group. Western blots were performed using 50 μg of total protein. GATA4 was detected with rabbit polyclonal antibody (Santa Cruz) and normalized to GAPDH (Research Diagnostics).

Results

Generation of gene-targeted mice

To circumvent the extracardiac early embryonic lethality seen in GATA4 null embryos, we generated a floxed GATA4 allele (Fig. 1a). A targeting construct was used to modify the GATA4 locus in embryonic stem cells by homologous recombination, yielding the allele *GATA4^{H}*. The targeted allele contains a loxP site in the 5′untranslated region of exon 2, 468 bp upstream of the initiation codon, and a neomycin resistance cassette in the second intron. The loxP site was oriented so that no ATG sequence was introduced in the 5′ untranslated region. Correct targeting was verified by Southern blotting (Fig. 1b) and PCR (not shown). Properly targeted ES cells were used to generate gene-targeted mice. GATA4$^{wt/H}$ mice were mated to EIIaCre mice (Williams-Simons and Westphal, 1999) to delete exon 2 in the germline, creating the allele *GATA4$^{\Delta}$* (Fig. 1a), which is similar in structure to a previously described null allele (Kuo et al., 1997). To delete the resistance cassette, GATA4$^{wt/H}$ mice were mated to hactB::FLPe transgenic mice (Rodriguez et al., 2000), giving the allele *GATA4^{flox}* (Fig. 1a).

Detailed analysis of each of these alleles indicated that *GATA4$^{\Delta}$* is a null allele, while *GATA4^{H}* is a severe and *GATA4^{flox}* a mild hypomorphic allele. Analysis of embryos from timed matings between GATA4$^{wt/\Delta}$ mice showed that GATA4$^{\Delta/\Delta}$ embryos did not survive beyond E10.5 (Fig. 2). Mutant embryos displayed severe abnormalities of ventral folding (data not shown), consistent with previously described GATA4 null alleles (Kuo et al., 1997; Molkentin et al., 1997). Intercrosses between GATA4$^{wt/H}$ mice did not produce any live GATA4$^{H/H}$ offspring (0/167), and analysis of litters from timed matings showed that GATA4$^{H/H}$ embryos died between E13.5–E16.5 (Fig. 2). These findings demonstrated that *GATA4^{H}* is a hypomorphic allele. Removal of the neomycin resistance cassette resulted in *GATA4^{flox}*, which is compatible with normal survival since intercrosses between GATA4$^{wt/flox}$ mice produced GATA4$^{flox/flox}$ offspring at the expected Mendelian frequency (32/132, 24%; Fig. 2).

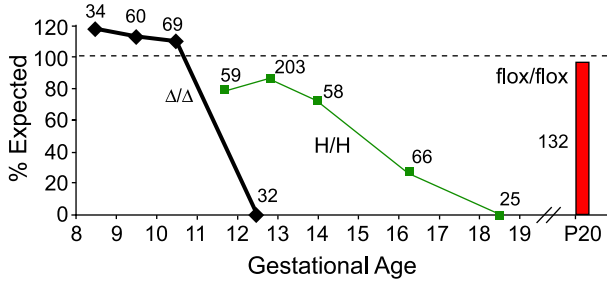

Fig. 2. Survival of offspring homozygous for modified GATA4 alleles. The graph displays the frequency of offspring with the indicated genotype from heterozygous intercrosses. The number of offspring genotyped for each point is indicated. P20, postnatal day 20.

Reduced GATA4 protein expression from GATA4^{H}

To determine the reason that the *GATA4^{H}* allele fails to support normal survival, we compared expression of GATA4 mRNA and protein in hearts from E13.5 GATA4$^{H/H}$ embryos and wild-type littermates. In situ hybridization revealed that GATA4 transcripts were normally distributed in GATA4$^{H/H}$ hearts (Fig. 3a). In both wild-type and mutant embryos, GATA4 is expressed at highest levels in the endocardium of the atrioventricular canal and in the atrioventricular endocardial cushions (Figs. 3a and b). Robust GATA4 expression was also present in wild-type and mutant myocardium (Figs. 3a and b). In E10.5 embryos, GATA4 was expressed similarly in the proepicardial derivatives of both wild-type and mutant hearts (data not shown). By E14, the epicardium is relatively thin and expression in this structure is difficult to resolve from myocardial expression by radioactive in situ hybridization. In the atrioventricular grooves, where epicardial cells tend to be heaped up, epicardial GATA4 expression was detected in both wild-type and mutant embryos (Fig. 3b). By Northern blotting, GATA4 transcripts from mutant hearts were normal in size and 30% reduced in abundance (Fig. 3c, left panel). qRT-PCR showed that GATA4 mRNA levels were normal in GATA4$^{flox/flox}$ hearts but 50% reduced in GATA4$^{H/H}$ hearts (Fig. 3c, right panel). Sequencing of GATA4 cDNA fragments amplified from GATA4$^{H/H}$ hearts demonstrated that the coding region was identical to wild type, and no aberrant splice variants could be detected by PCR using primers spanning each intron–exon junction.

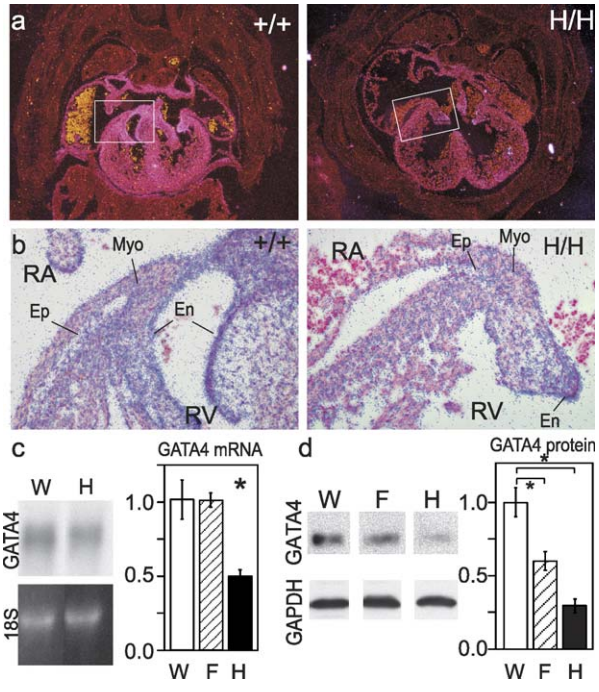

Fig. 3. GATA4 mRNA and protein expression in GATA4 mutant embryos. (a) Dark-field images demonstrating GATA4 expression (pink) in GATA4$^{H/H}$ and wild-type hearts, detected by in situ hybridization using a full-length GATA4 probe. White box indicates area magnified in panel b. (b) Higher magnification of the right atrioventricular groove. To facilitate anatomical localization of the GATA4 in situ hybridization signal, it is displayed as blue pseudocolor overlaid on the bright-field eosin-stained image. GATA4 is expressed in endocardium (En), myocardium (Myo), and epicardium (Ep). RA, right atrium; RV, right ventricle. (c) GATA4 mRNA from E13.5 GATA4$^{H/H}$ and GATA4$^{+/+}$ hearts. GATA4 transcript was analyzed by Northern blotting using a full-length GATA4 probe, and by quantitative RT-PCR, normalized to 18 S rRNA. *$P < 0.05$. (d) GATA4 protein in mutant and wild-type E13.5 hearts. GATA4 protein from H/H, flox/flox, and wild-type E13.5 hearts was analyzed by Western blotting. Each sample was pooled from two hearts. (d) Quantitative analysis of GATA4 protein expression, normalized to GAPDH. Each value represents the average of three samples, each pooled from two hearts. *$P < 0.05$. W, wild-type; F, GATA4$^{flox/flox}$; H, GATA4$^{H/H}$.

Table 1
Cardiac morphology of GATA4$^{H/H}$

	+/+	GATA4$^{H/H}$
n	18	18
Normal	17 (94)	0
Atria		
RA dilatation	0	7 (39)
AV Junction		
CAVC	0	11[a] (61)
Partial AVC	0	1[b] (6)
Ventricles		
Spongy septum	0	10 (56)
Hypoplasia	0	18 (100)
Inlet VSD	0	12 (67)
Membranous VSD	1 (6)	2[c] (11)
Musc VSD	0	2 (11)
Outflow		
DORV	0	16 (89)

[a] Two unbalanced canal, LV dominant.
[b] No primum ASD.
[c] Membranous VSDs with extension into the inlet septum.

By Western blotting, GATA4 protein expressed from the *GATA4^{H}* allele was normal in size (Fig. 3d). The amount of GATA4 protein, normalized to GAPDH, was reduced by 70% in GATA4$^{H/H}$ hearts (Fig. 3d). In GATA4$^{flox/flox}$ hearts, GATA4 protein was present at an intermediate level (50% reduction compared to wild-type controls; Fig. 2c). This small difference in GATA4 protein levels was associated with a profound difference in embryonic survival (Fig. 2).

Abnormal cardiac morphogenesis in GATA4$^{H/H}$ embryos

By E13.5, GATA4$^{H/H}$ embryos appeared edematous and the right atrium was dilated, displacing the heart to the left (Figs. 4a and b). Mutant hearts had common atrioventricular canal (CAVC), usually well balanced but occasionally with a relatively small right ventricle (Fig. 4b; Table 1). The interventricular septum had a characteristic spongiform appearance (Fig. 4b; Table 1), and the compact and trabecular myocardium were markedly hypoplastic (Figs. 4b and 5a and b). The aorta was connected with the right ventricle, resulting in a double outlet right ventricle (Figs. 4b and c). A subaortic

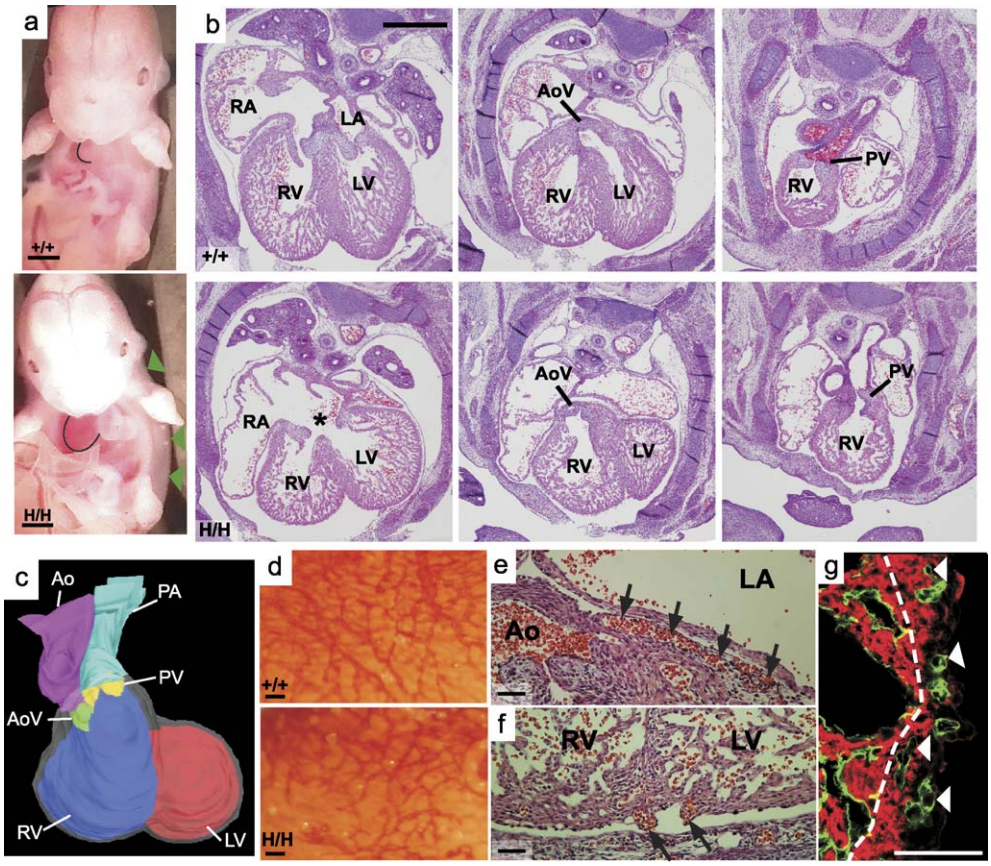

Fig. 4. Morphology of GATA4$^{H/H}$ embryos. (a) Mutant embryos had body wall edema (green arrowheads) and dilated right atria (outlined in black), displacing the heart to the left. Scale bar = 1 mm. (b) Serial hematoxylin-and-eosin-stained transverse sections of hearts from wild-type and mutant E14.5 littermate embryos. The GATA4H/H heart demonstrated CAVC (*, left panel), hypoplasia of the compact and trabecular myocardium, and dilated right atrium. The aortic (middle panel) and the pulmonary valves (right panel) both connected to the right ventricle in the mutant embryo. Scale bar = 0.5 mm. (c) Three-dimensional reconstruction of a GATA4$^{H/H}$ heart showing DORV. Both semilunar valves were related exclusively to the right ventricle, with the aortic valve inferior, posterior, and to the right of the pulmonary valve. (d) Dorsal surface of wild-type and GATA4$^{H/H}$ littermate ventricles at E13.5. Coronary vasculature was identified by whole mount PECAM staining. No difference in staining was seen between wild-type and mutant hearts. (e) Blood-filled left main coronary artery (arrows) in a GATA4$^{H/H}$ heart. (f) Blood-filled small epicardial coronary arteries (arrows) in the interventricular groove of a GATA4$^{H/H}$ heart. (g) Intramyocardial coronary arteries (arrowheads) in a GATA4$^{H/H}$ heart. Green, PECAM; red, desmin. Dashed white line indicates inner margin of the compact myocardium; d–g, scale bar = 0.05 mm. RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle; AoV, aortic valve; PV, pulmonary valve; Ao, aorta.

conus was present (not shown), and the aortic valve was inferior, posterior, and to the right of the pulmonary valve (Fig. 4c).

A previously described GATA4 hypomorphic allele, *GATA4^{Ki}*, expressed normal amounts of a mutant GATA4 protein that failed to interact with FOG2 due to a V217G mutation. GATA4$^{Ki/Ki}$ embryos died around E12.5, and like GATA4$^{H/H}$ hearts, GATA4$^{Ki/Ki}$ hearts had CAVC, DORV, and ventricular hypoplasia. GATA4$^{Ki/Ki}$ hearts had a striking paucity of coronary vasculature (Crispino et al., 2001). In contrast, we found that GATA4$^{H/H}$ hearts had normal, blood-filled epicardial coronary vessels (Figs. 4d–f). PECAM staining demonstrated the presence of intramyocardial coronary vessels (Fig. 4g).

Hypoplasia of the compact and trabecular myocardium in GATA4$^{H/H}$ embryos (Figs. 5a and b) may have been due to increased cardiomyocyte apoptosis or decreased cardiomyocyte proliferation. TUNEL staining of E12.5 and E13.5 hearts did not reveal a difference in the rate of apoptosis between mutant embryos and wild-type littermate controls (data not shown). However, cardiomyocyte proliferation, as assessed by BrdU labeling, was significantly decreased in GATA4$^{H/H}$ compact myocardium compared to wild-type littermate controls at E12.5 (Figs. 5c and d). While mutant hearts also had marked hypoplasia of the trabecular myocardium, we did not detect a difference in the frequency of BrdU-positive cells in the trabecular myocardium at E12.5 (Fig. 5d).

Trabecular myocardium and compact myocardium have distinct gene expression programs. In normal hearts, at E13.5 *ANF* was expressed at high levels in trabecular but not compact myocardium (Fig. 5e), while *N-myc* was expressed at higher levels in compact compared to trabecular myocardium (Fig. 5f). The outer margin of *ANF* expression and the inner margin of *N-myc* expression (dashed line, Figs. 5e–f) coincided with the morphological junction between compact and trabecular myocardium (solid

Table 2
Hemodynamic measurements in E13.5 embryos

	WT	H/H	*p*
Video microscopy			
n	*13*	*12*	
HR	200 ± 3	225 ± 10	<0.05
LVEDA (mm^2)	0.78 ± 0.01	0.79 ± 0.01	
LVESA (mm^2)	0.52 ± 0.02 (13)	0.52 ± 0.01	
LVAEF (%)	34.8 ± 1.3	34.8 ± 0.9	
RVEDA (mm^2)	1.27 ± 0.03	1.27 ± 0.03	
RVESA (mm^2)	0.83 ± 0.02	0.81 ± 0.02	
RVAEF (%)	33.9 ± 0.67	36.4 ± 1.33	
Doppler velocimetry			
n	*11*	*10*	
E (mm/s)	25 ± 1	52 ± 3	<0.001
A (mm/s)	97 ± 2	103 ± 5	
E/A	0.26 ± 0.00	0.50 ± 0.03	<0.001
Intraventricular pressure			
n	*7*	*4*	
Peak (mm Hg)	9.00 ± 0.40	6.75 ± 0.16	<0.005
EDP (mm Hg)	0.79 ± 0.03	1.05 ± 0.06	<0.005
A kick (mm Hg)	2.05 ± 0.04	1.90 ± 0.12	
Sucking (mm Hg)	−0.60 ± 0.03	−0.15 ± 0.09	<0.001
+dP/dt (mm Hg/s)	217 ± 8	196 ± 9	
−dP/dt (mm Hg/s)	185 ± 3	155 ± 5	<0.001

LV, left ventricle; RV, right ventricle; EDA, end diastolic area; ESA, end systolic area; AEF, area ejection fraction; EDP, end diastolic pressure.

line, Figs. 5e–f). In $GATA4^{H/H}$ embryos, *ANF* continued to be expressed at high levels in trabecular myocardium, but the outer margin of *ANF* expression (dashed line) occurred in a region of myocardium with a trabecular appearance (Fig. 5e). Similarly, the inner margin of *N-myc* expression (dashed line) occurred in a region of myocardium with a trabecular appearance (Fig. 5f). These data indicate that in mutant embryos there was a region of dysplastic myocardium that expressed the genetic program of compact myocardium but had trabecular morphology.

Impaired diastolic function Of $GATA4^{H/H}$ hearts

To determine the effect of the hypomorphic GATA4 mutation on embryo cardiac function, we used video imaging, Doppler velocimetry, and intracardiac pressure measurements to evaluate the hemodynamics of E13.5 embryos (Fig. 6; Table 2). We found that ventricular size was no different between mutant and wild-type embryos (Table 2). Systolic function, as assessed by area ejection fraction and +dP/dt, was also normal in $GATA4^{H/H}$ embryos, although peak ventricular pressure was reduced in mutant embryos (Table 2), perhaps as a result of atrioventricular regurgitation (see below). Diastolic function, as assessed by the ratio of early ("E") to late ("A") ventricular inflow velocities, −dP/dt, ventricular end-diastolic pressure, and ventricular sucking pressure, was severely impaired in $GATA4^{H/H}$ embryos (Table 2). We also qualitatively assessed competency of the atrioventricular (AV) valves by measuring blood flow in the inferior vena cava. We detected systolic retrograde flow in mutant embryos, indicating that the hypoplastic AV valves of mutant hearts were regurgitant (Fig. 6c).

Gene expression in $GATA4^{H/H}$ hearts

To determine if chamber- and subregion-specific genetic programs were normally specified in $GATA4^{H/H}$ embryos, we used in situ hybridization to detect subregion-restricted transcripts. *MLC2a* and *MLC2v*, which are restricted to atrial and ventricular myocardium, respectively, were normally expressed in $GATA4^{H/H}$ hearts (data not shown). *Tbx5* retained its normal preferential expression in the atria and the left ventricle (data not shown).

Based on promoter assays in transfected cultured cells and in some cases promoter studies in transgenic animals, GATA4 has been implicated in the regulation of a number of genes expressed in the heart. To determine if variation of GATA4 expression levels within a physiologically relevant range influences expression of putative GATA4 target genes, we measured the expression of selected genes by quantitative RT-PCR (Fig. 7). *GATA6* expression was not altered in $GATA4^{H/H}$ embryos, in contrast to the compensatory up-regulation of *GATA6* reported in GATA4 null embryos (Molkentin et al., 1997). Although in vivo promoter analysis indicated that GATA sites were essential for normal activity of the *Nkx2-5* and *MEF2C* enhancers (Lien et al., 1999; Searcy et al., 1998; B. Black, personal communication), *Nkx2-5* levels were up-regulated twofold in $GATA4^{H/H}$ hearts while *MEF2C* expression was unchanged (Fig. 7). Likewise, *ANF* and *BNP* expression levels were not altered in $GATA4^{H/H}$ hearts (Fig. 7) despite the extensive literature suggesting an important role for GATA4 in the activation of these promoters. Because of the reduced replication rate of $GATA4^{H/H}$ myocytes, we also measured expression of *cyclin D1–3* and the homeodomain only protein (*HOP*), which regulates myocardial growth and is a direct target of the GATA4 interacting transcription factor *Nkx2-5* (Chen et al., 2002; Shin et al., 2002). *Cyclin D2*, *cyclin D3*, and *HOP* expression were not significantly changed in E12.5 mutant hearts, while *cyclin D1* expression was slightly up-regulated in mutant hearts (Fig. 7). Finally, we examined the expression of *BMP4* because *BMP4* has been reported to be a target of *GATA4* and atrioventricular canal formation is sensitive to *BMP4* dosage. We did not detect a difference in *BMP4* expression by in situ hybridization in E10.5 hearts (data not shown) or by RT-PCR in E13.5 hearts (Fig. 7).

Discussion

GATA4 regulation of cardiac morophogenesis

We have generated and characterized a hypomorphic allele of GATA4, *$GATA4^{H}$*. $GATA4^{H/H}$ embryos expressed 70% lower levels of GATA4 protein (Fig. 3). These mutant

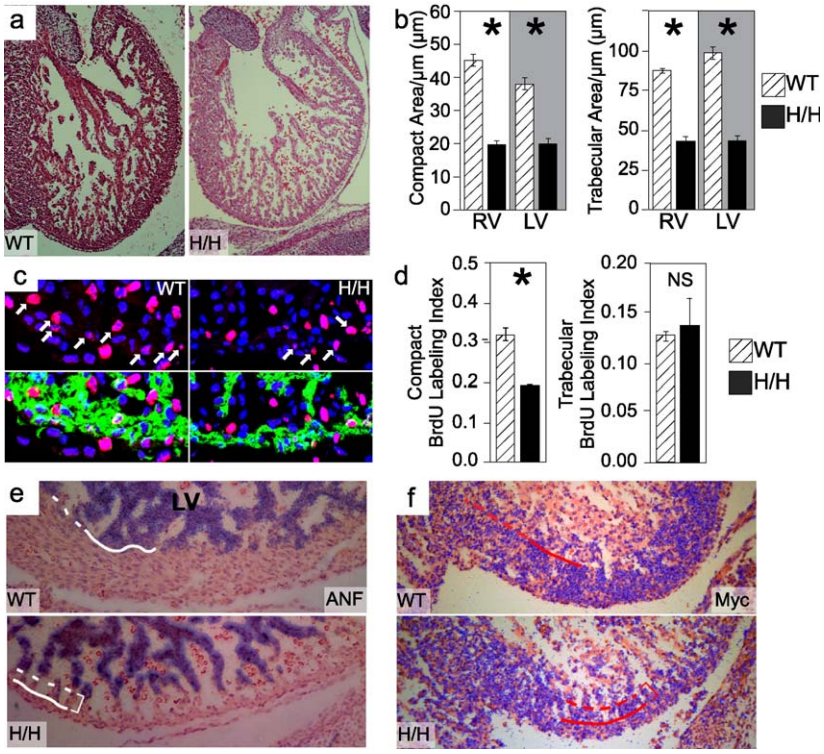

Fig. 5. Hypoplasia and dysplasia of ventricular myocardium in GATA4$^{H/H}$ hearts. (a) Left ventricle of mutant and wild-type hearts at the level of the major papillary muscles. (b) Quantitative analysis of compact and trabecular areas, normalized to ventricular circumference. The compact and trabecular areas of mutant RV and mutant LV were significantly reduced compared to littermate controls ($n = 5$; *$P < 0.001$). (c) BrdU staining (red) of GATA4$^{H/H}$ and wild-type left ventricle at E12.5. Nuclei were detected by TOPRO-3 staining (blue). Lower panels are the same images as the upper panels, with cardiomyocytes identified by desmin staining (green). Arrows indicate BrdU-positive cardiomyocyte nuclei. (d) Quantitative analysis of cell replication in E12.5 mutant and wild-type hearts. BrdU labeling index was calculated as the ratio of BrdU-positive cardiomyocyte nuclei to total cardiomyocyte nuclei. More than 200 nuclei were counted for each measurement per embryo. Mutant embryos were compared to their respective wild-type littermates. Each group contained three embryos. *$P < 0.05$. NS, no significant difference. (e) Abnormal regional expression of *ANF* in mutant hearts. In control hearts, *ANF* (blue in situ hybridization signal) was expressed in trabecular myocardium all the way to the junction with compact myocardium (solid line). In mutant hearts, the *ANF* expression domain (dashed line) did not extend to the morphological compact myocardium. The bracket indicates a region with trabecular appearance but lacking ANF expression. (f) Abnormal regional expression of *N-myc* in mutant hearts. In control hearts, *N-myc* was most highly expressed in compact myocardium (blue in situ hybridization signal). In mutant hearts, the domain of higher level *N-myc* expression (dashed line) extended beyond the morphological compact myocardium (solid line).

hearts had CAVC, DORV, and myocardial hypoplasia (Fig. 4). GATA4$^{H/H}$ embryos died between E13.5 and E16.5, with right atrial dilatation and peripheral edema suggestive of congestive heart failure. In comparison, GATA4$^{flox/flox}$ embryos, which had a 50% reduction of GATA4 protein (Fig. 3), survived normally (Fig. 2) and had normal postnatal cardiac function and fertility (WTP and SI, unpublished data). These data indicate that small changes in the level of GATA4 protein expression can dramatically influence cardiac morphogenesis and embryonic survival. Consistent with this conclusion is the association of mutations of GATA4 with cardiac septation defects in humans (Garg et al., 2003). The extent to which the *GATA4^{H}* allele reduced GATA4 expression is within the range of GATA4 down-regulation that has been seen with experimental retinoic acid deficiency (Kostetskii et al., 1999) and with perturbation of embryonic calcium homeostasis (Porter et al., 2003), suggesting that altered GATA4 activity may contribute to congenital heart disease due to both genetic and environmental factors.

The morphology of GATA4$^{Ki/Ki}$ hearts was similar to GATA4$^{H/H}$ hearts, with both having CAVC, DORV, and marked hypoplasia of the compact myocardium (Crispino et al., 2001). This similarity indicates that many dosage-sensitive aspects of GATA4 function in late cardiac morphogenesis are dependent upon interaction with a FOG cofactor. Both GATA4$^{Ki/Ki}$ and GATA4$^{H/H}$ hearts have striking myocardial hypoplasia (Fig. 5). The hypoplasia of the compact myocardium in GATA4$^{H/H}$ embryos was due at least in part to decreased cardiomyocyte replication at E12.5. Although we did not find a difference in BrdU labeling index in trabecular myocardium at E12.5 or E13.5, it is possible that earlier time points would have to be examined since the most rapid rate of growth of trabecular myocardium precedes E12.5 (Ishiwata et al., 2003). GATA factors have been implicated in the regulation of cell proliferation in other systems, potentially through the regulation of *cyclin D2* (Kitta et al., 2003; Suzuki et al., 2003; Tanaka et al., 2000; Wang et al., 1996). However, we found that transcription of *cyclins D1–3* was unaltered or

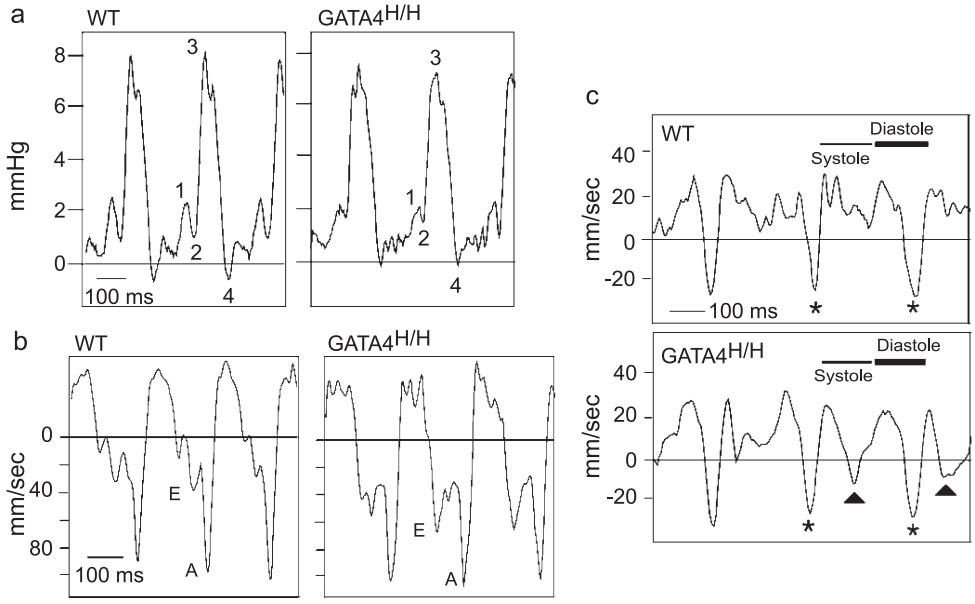

Fig. 6. Analysis of embryo hemodynamics. (a) Representative intraventricular pressure tracings from wild-type and mutant embryos. (1) Atrial kick pressure; (2) EDP; (3) peak systolic pressure; (4) sucking pressure. (b) Representative Doppler recordings of ventricular inflow velocities. E—early diastolic filling; A—late diastolic filling. (c) Representative Doppler recordings of inferior vena cava flow. There is normally a brief wave of retrograde flow (*) during atrial contraction (late ventricular diastole). Abnormal systolic retrograde flow, indicated by arrowheads, was detected in all mutant embryos but in no wild-type embryos.

slightly up-regulated in GATA4$^{H/H}$ embryos (Fig. 7). Likewise, transcription of *HOP*, a regulator of myocardial growth and a target gene of the GATA4 interacting transcription factor Nkx2-5 (Chen et al., 2002; Shin et al., 2002), was not changed in GATA4$^{H/H}$ embryos (Fig. 7).

While the overall phenotype of GATA4$^{Ki/Ki}$ and GATA4$^{H/H}$ hearts was similar, unlike GATA4$^{Ki/Ki}$ hearts GATA4$^{H/H}$ hearts had blood filled epicardial and intramyocardial coronary vessels (Figs. 4d–g). This difference suggests that coronary vessel formation requires GATA4-FOG interaction but is not sensitive to a moderate reduction in GATA4 levels. This implies that GATA4 is necessary for coronary vessel formation but might not be a dose-limiting regulator of this process. Although our analysis cannot completely exclude abnormal perfusion due to abnormal coronary vessel density or microvascular function, the equivalent severity of myocardial hypoplasia despite a marked difference in coronary vascularity in the GATA4$^{Ki/Ki}$ versus GATA4$^{H/H}$ mutant hearts suggests that the myocardial hypoplasia is not secondary to abnormal perfusion.

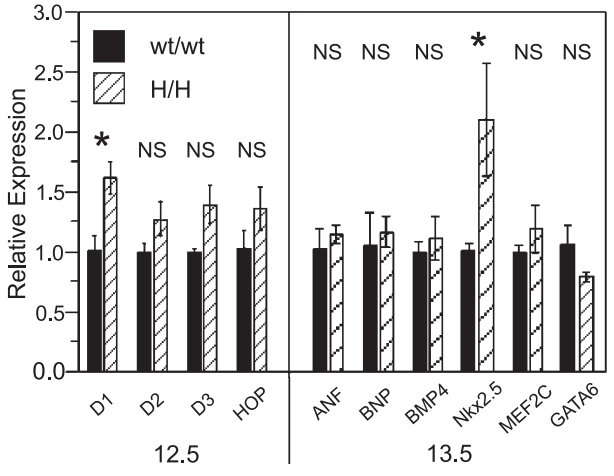

Fig. 7. Gene expression in GATA4$^{H/H}$ hearts. Relative expression of GATA4 target genes, normalized to GAPDH (ANF, BNP) or 18 S, in wild-type and H/H hearts. Expression was measured by qRT-PCR using total RNA from E13.5 hearts. *$P < 0.05$. NS, no significant difference. Three samples, each composed of hearts from two embryos, were used for each group and each measurement was made in duplicate.

We found that the expression domains of *ANF* and *N-myc*, markers that delineate the boundary between compact and trabecular myocardium, were perturbed in mutant embryos (Figs. 4e–f) so that mutant embryos had a zone with the morphology of trabecular myocardium but the genetic program of compact myocardium (reduced *ANF* expression, increased *N-myc* expression). This phenotype is reminiscent of ventricular noncompaction, a frequent finding in childhood cardiomyopathy (Nugent et al., 2003) and a finding associated with a chromosomal microdeletion that includes GATA4 (Pehlivan et al., 1999). The thin myocardial phenotype of GATA4$^{H/H}$ hearts suggests that GATA4 and its transcriptional targets are candidate genes for human cardiomyopathies.

GATA4 expression in ventral endoderm has previously been demonstrated to be sufficient to rescue the defect of ventral morphogenesis seen in GATA4 null embryos (Narita et al., 1997). GATA4$^{H/H}$ embryos undergo normal ventral morphogenesis, suggesting that either the requirement for GATA4 is less sensitive to expression level in the ventral endoderm compared to the heart or that the *GATA4^{H}* allele

expresses higher amounts of GATA4 in the ventral endoderm than in the heart.

Hemodynamic abnormalities leading embryonic lethality

Like FOG2$^{\Delta/\Delta}$ embryos (Ishiwata et al., 2003), GATA4$^{H/H}$ embryos had severely diminished ventricular diastolic function and normal systolic function (Fig. 6). While it might seem counterintuitive that the thinner mutant ventricle was stiffer than wild-type ventricle, diastolic ventricular filling is an active process. The compact myocardium expresses relatively higher levels the calcium handling proteins SERCA2A, phospholambam, and sodium–calcium exchanger (NCX1) than the trabecular myocardium, and early ventricular filling is exponentially related to the area of the compact myocardium (Ishiwata et al., 2003). Our finding that the thinner walled mutant myocardium had impaired diastolic function is consistent with these data. In addition to their effects on myoarchitecture, GATA4 and FOG2 may also influence diastolic function by altering the expression of cardiac genes that are important for ventricular relaxation.

Our hemodynamic measurements describe the pathophysiology leading to death in GATA4$^{H/H}$ embryos. During systole, a fraction of the ventricular output regurgitated retrograde due to incompetence of the hypoplastic atrioventricular valves. In the setting of AV regurgitation, normal rather than supranormal area ejection fraction suggests the possibility that the mutant embryos also had latent systolic dysfunction. During diastole, the regurgitant volume did not reenter the ventricle (EDA was normal rather than increased in mutant embryos) because of impaired ventricular relaxation. As a result, diastolic pressures were elevated, leading to atrial enlargement and peripheral edema in mutant embryos (Fig. 4). Moreover, forward cardiac output was reduced, resulting in death of the embryo. We suspect that impaired ventricular filling is an important component of the hemodynamic derangements present in other embryos with hypoplasia of the compact myocardium.

Regulation of GATA4 downstream targets

GATA4 has been implicated in the regulation of a large number of cardiac genes (reviewed by Molkentin, 2000), based largely on reporter assays in cell culture or in transgenic animals. We tested the hypothesis that GATA4 is a dosage-limiting regulator of transcription of a number of putative target genes. While *ANF*, *BNP*, *cyclin D2*, and *BMP4* promoters are GATA4 responsive in transient transfection assays, we found that decreased GATA4 protein levels did not alter expression of these genes in vivo (Figs. 5e and 7), suggesting that changes in GATA4 level within a physiological range do not alter the expression of these genes in late cardiogenesis. Consistent with this finding, *ANF* was normally expressed in GATA4 null hearts (Molkentin et al., 1997). However, we cannot exclude more complex models in which down-regulation resulting from decreased GATA4 protein levels is masked by up-regulation due to other factors, such as heart failure.

The enhancers of the transcription factors *Nkx2-5* and *MEF2C* contain GATA4 binding sites, and mutation of these sites altered expression of these genes in transgenic reporter mice (Lien et al., 1999; Searcy et al., 1998; B. Black, personal communication). However, *MEF2C* and *Nkx2.5* transcript levels were unaffected or twofold up-regulated, respectively, in GATA4$^{H/H}$ hearts, indicating that the expression of these genes is not sensitive to a moderate reduction of GATA4 activity late in cardiogenesis. This does not exclude an important role of GATA4 in inducing or maintaining the expression of these genes at other stages of development. The up-regulation of *Nkx2-5* in GATA4$^{H/H}$ hearts was unexpected and may be secondary to the increased wall stress in mutant hearts. *Nkx2-5* was up-regulated by increased wall stress in adult hearts (Thompson et al., 1998).

Conclusion

GATA4 is an essential, dosage-dependent regulator of cardiac morphogenesis. A threshold level of GATA4 between 30% and 50% of normal is required for normal heart development and embryonic survival. Reduction of GATA4 protein below this threshold resulted in reduced cardiomyocyte replication, myocardial hypoplasia, and endocardial cushion defects.

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References

Basson, C.T., Bachinsky, D.R., Lin, R.C., Levi, T., Elkins, J.A., Soults, J., Grayzel, D., Kroumpouzou, E., Traill, T.A., Leblanc-Straceski, J., Renault, B., Kucherlapati, R., Seidman, J.G., Seidman, C.E., 1997. Mutations in human TBX5 cause limb and cardiac malformation in Holt-Oram syndrome. Nat. Genet. 15, 30–35.

Biben, C., Weber, R., Kesteven, S., Stanley, E., McDonald, L., Elliott, D.A., Barnett, L., Koentgen, F., Robb, L., Feneley, M., Harvey, R.P., 2000. Cardiac septal and valvular dysmorphogenesis in mice heterozygous for mutations in the homeobox gene *Nkx2-5*. Circ. Res. 87, 888–895.

Bruneau, B.G., Nemer, G., Schmitt, J.P., Charron, F., Robitaille, L., Caron, S., Conner, D.A., Gessler, M., Nemer, M., Seidman, C.E., Seidman, J.G., 2001. A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. Cell 106, 709–721.

Chen, F., Kook, H., Milewski, R., Gitler, A.D., Lu, M.M., Li, J., Nazarian, R., Schnepp, R., Jen, K., Biben, C., Runke, G., Mackay, J.P., Novotny, J., Schwartz, R.J., Harvey, R.P., Mullins, M.C., Epstein, J.A., 2002. Hop is an unusual homeobox gene that modulates cardiac development. Cell 110, 713–723.

Crispino, J.D., Lodish, M.B., Thurberg, B.L., Litovsky, S.H., Collins, T., Molkentin, J.D., Orkin, S.H., 2001. Proper coronary vascular development and heart morphogenesis depend on interaction of GATA-4 with FOG cofactors. Genes Dev. 15, 839–844.

Garg, V., Kathiriya, I.S., Barnes, R., Schluterman, M.K., King, I.N., Butler, C.A., Rothrock, C.R., Eapen, R.S., Hirayama-Yamada, K., Joo, K., Matsuoka, R., Cohen, J.C., Srivastava, D., 2003. GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. Nature 424, 443–447.

Hoffman, J.I., 1995. Incidence of congenital heart disease: I. Postnatal incidence. Pediatr. Cardiol. 16, 103–113.

Ishiwata, T., Nakazawa, M., Pu, W.T., Tevosian, S.G., Izumo, S., 2003. Developmental changes in ventricular diastolic function correlate with changes in ventricular myoarchitecture in normal mouse embryos. Circ. Res. 93, 857–865.

Kitta, K., Day, R.M., Kim, Y., Torregroza, I., Evans, T., Suzuki, Y.J., 2003. Hepatocyte growth factor induces GATA-4 phosphorylation and cell survival in cardiac muscle cells. J. Biol. Chem. 278, 4705–4712.

Kostetskii, I., Jiang, Y., Kostetskaia, E., Yuan, S., Evans, T., Zile, M., 1999. Retinoid signaling required for normal heart development regulates GATA-4 in a pathway distinct from cardiomyocyte differentiation. Dev. Biol. 206, 206–218.

Kuo, C.T., Morrisey, E.E., Anandappa, R., Sigrist, K., Lu, M.M., Parmacek, M.S., Soudais, C., Leiden, J.M., 1997. GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. Genes Dev. 11, 1048–1060.

Lien, C.L., Wu, C., Mercer, B., Webb, R., Richardson, J.A., Olson, E.N., 1999. Control of early cardiac-specific transcription of *Nkx2-5* by a GATA-dependent enhancer. Development 126, 75–84.

Molkentin, J.D., 2000. The zinc finger-containing transcription factors GATA-4, -5, and -6. Ubiquitously expressed regulators of tissue-specific gene expression. J. Biol. Chem. 275, 38949–38952.

Molkentin, J.D., Lin, Q., Duncan, S.A., Olson, E.N., 1997. Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. Genes Dev. 11, 1061–1072.

Narita, N., Bielinska, M., Wilson, D.B., 1997. Wild-type endoderm abrogates the ventral developmental defects associated with GATA-4 deficiency in the mouse. Dev. Biol. 189, 270–274.

Nugent, A.W., Daubeney, P.E., Chondros, P., Carlin, J.B., Cheung, M., Wilkinson, L.C., Davis, A.M., Kahler, S.G., Chow, C.W., Wilkinson, J.L., Weintraub, R.G., 2003. The epidemiology of childhood cardiomyopathy in Australia. N. Engl. J. Med. 348, 1639–1646.

Pehlivan, T., Pober, B.R., Brueckner, M., Garrett, S., Slaugh, R., Van Rheeden, R., Wilson, D.B., Watson, M.S., Hing, A.V., 1999. GATA4 haploinsufficiency in patients with interstitial deletion of chromosome region 8p23.1 and congenital heart disease. Am. J. Med. Genet. 83, 201–206.

Porter Jr., G.A., Makuck, R.F., Rivkees, S.A., 2003. Intracellular calcium plays an essential role in cardiac development. Dev. Dyn. 227, 280–290.

Rodriguez, C.I., Buchholz, F., Galloway, J., Sequerra, R., Kasper, J., Ayala, R., Stewart, A.F., Dymecki, S.M., 2000. High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. Nat. Genet. 25, 139–140.

Schott, J.J., Benson, D.W., Basson, C.T., Pease, W., Silberbach, G.M., Moak, J.P., Maron, B.J., Seidman, C.E., Seidman, J.G., 1998. Congenital heart disease caused by mutations in the transcription factor NKX2-5. Science 281, 108–111.

Searcy, R.D., Vincent, E.B., Liberatore, C.M., Yutzey, K.E., 1998. A GATA-dependent *nkx-2.5* regulatory element activates early cardiac gene expression in transgenic mice. Development 125, 4461–4470.

Shin, C.H., Liu, Z.P., Passier, R., Zhang, C.L., Wang, D.Z., Harris, T.M., Yamagishi, H., Richardson, J.A., Childs, G., Olson, E.N., 2002. Modulation of cardiac growth and development by HOP, an unusual homeodomain protein. Cell 110, 725–735.

Srivastava, D., Olson, E.N., 2000. A genetic blueprint for cardiac development. Nature 407, 221–226.

Suzuki, Y.J., Day, R.M., Tan, C.C., Sandven, T.H., Liang, Q., Molkentin, J.D., Fanburg, B.L., 2003. Activation of GATA-4 by serotonin in pulmonary artery smooth muscle cells. J. Biol. Chem. 278, 17525–17531.

Tanaka, M., Chen, Z., Bartunkova, S., Yamasaki, N., Izumo, S., 1999. The cardiac homeobox gene Csx/*Nkx2.5* lies genetically upstream of multiple genes essential for heart development. Development 126, 1269–1280.

Tanaka, H., Matsumura, I., Nakajima, K., Daino, H., Sonoyama, J., Yoshida, H., Oritani, K., Machii, T., Yamamoto, M., Hirano, T., Kanakura, Y., 2000. GATA-1 blocks IL-6-induced macrophage differentiation and apoptosis through the sustained expression of cyclin D1 and bcl-2 in a murine myeloid cell line M1. Blood 95, 1264–1273.

Tevosian, S.G., Deconinck, A.E., Tanaka, M., Schinke, M., Litovsky, S.H., Izumo, S., Fujiwara, Y., Orkin, S.H., 2000. FOG-2, a cofactor for GATA transcription factors, is essential for heart morphogenesis and development of coronary vessels from epicardium. Cell 101, 729–739.

Thompson, J.T., Rackley, M.S., O'Brien, T.X., 1998. Up-regulation of the cardiac homeobox gene *Nkx2-5* (CSX) in feline right ventricular pressure overload. Am. J. Physiol. 274, H1569–H1573.

Wang, Z., Sicinski, P., Weinberg, R.A., Zhang, Y., Ravid, K., 1996. Characterization of the mouse cyclin D3 gene: exon/intron organization and promoter activity. Genomics 35, 156–163.

Williams-Simons, L., Westphal, H., 1999. EIIaCre—Utility of a general deleter strain. Transgenic Res. 8, 53–54.

Zeller, R., Bloch, K.D., Williams, B.S., Arceci, R.J., Seidman, C.E., 1987. Localized expression of the atrial natriuretic factor gene during cardiac embryogenesis. Genes Dev. 1, 693–698.