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Original article

Spectrum of heart disease associated with murine and human *GATA4* mutation

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Abstract

The transcription factor GATA4 is essential for heart morphogenesis. Heterozygous mutation of *GATA4* causes familial septal defects. However, the phenotypic spectrum of heterozygous GATA4 mutation is not known. In this study, we defined the cardiac phenotypes that result from heterozygous mutation of murine *Gata4*. We then asked if *GATA4* mutation occurs in humans with these forms of congenital heart disease (CHD). In mice, heterozygous *Gata4* mutation was associated with atrial and ventricular septal defect (ASD, VSD), endocardial cushion defect (ECD), RV hypoplasia, and cardiomyopathy. Genetic background strongly influenced the expression of ECD and cardiomyopathy, indicating the presence of important genetic modifiers. In humans, non-synonymous *GATA4* sequence variants were associated with ECD (2/43), ASD (1/8), and RV hypoplasia in the context of double inlet left ventricle (1/9), forms of CHD that overlapped with abnormalities seen in the mouse model. These variants were not found in at least 500 control chromosomes, and encode proteins with non-conservative amino acid substitutions at phylogenetically conserved positions, suggesting that they are disease-causing mutations. Cardiomyopathy was not associated with *GATA4* mutation in humans. These data establish the phenotypic spectrum of heterozygous *Gata4* mutation in mice, and suggest that heterozygous *GATA4* mutation leads to partially overlapping phenotypes in humans. Additional studies will be required to determine the degree to which *GATA4* mutation contributes to human CHD characterized by ECD or RV hypoplasia.

Keywords: Animal models; Congenital heart defects; Cardiac development; Genetics of congenital heart disease

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1. Introduction

The morphogenetic complexity of fashioning a four-chambered heart from a straight tube mandates a precisely orchestrated interplay of multiple transcription factors, adhesion molecules, ion channels, signaling molecules and structural proteins [1]. Errors in this process result in congenital heart disease (CHD), the most common form of birth defect. Mutation of a small but growing number of genes has been shown to cause CHD [2]. Recently, mutation of the zinc finger transcription factor *GATA4* was shown to cause atrial and ventricular septal defects in several unrelated extended pedigrees (Table 1) [3–6]. Among CHD patients without a family history ("sporadic" CHD), *GATA4* mutations appear to be infrequent. In published studies of sporadic CHD, *GATA4* mutations were found in only 2 out of 376 probands examined (Table 1) [7–10]. However, without prior knowledge of the phenotypic spectrum of *GATA4* mutation, it was not possible to target these studies to forms of CHD most likely to be caused by *GATA4* mutation. Therefore *GATA4* mutation may be more frequently associated with specific forms of CHD that are not well represented in current literature.

We studied the spectrum of cardiac abnormalities found in mice with mutation of one copy of *GATA4*. We found that heterozygous *GATA4* mutation in mice caused endocardial cushion defect (ECD), atrial or ventricular septal defect (ASD or VSD), hypoplastic right ventricle, and cardiomyopathy. Reasoning that this might give insight into the types of heart defects that might be caused by *GATA4* mutation in humans, we then looked for GATA4 mutations in patients with similar forms of CHD. We found non-synonymous *GATA4* sequence variants in association with ECD, hypoplastic RV in the context of double inlet left ventricle (DILV), and ASD. These results refine our understanding of the phenotypic spectrum of *GATA4* mutation.

2. Methods

2.1. Mice

The $Gata4^{\Delta ex2}$ allele has been described [11]. Mice were backcrossed for more than 7 generations into either the C57BL6/J (abbreviated C57; Jackson Labs) or the FVB/NCrl (FVB; Charles River Labs) genetic backgrounds. Structural abnormalities were diagnosed on H&E-stained serial paraffin sections. Echocardiography was performed on unsedated 8-week-old mice. Mice were held in a supine position and imaged with a 15-MHz probe. All analyses were performed blinded to genotype. Animal use was according to protocols approved by the Institutional Animal Care and Use Committee, and conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

For the genetic modifier screen, mice were genotyped using the Illumina MD linkage panel. A whole genome linkage scan was performed with ECD as a binary trait, using the model of Xu and Atchley [12]. Genome scans were performed using the R/qtl package [13].

2.2. Patients and GATA4 genotyping

After obtaining informed consent, blood and/or saliva were obtained from probands, and when possible from members of their nuclear family. We studied 107 probands with cardiac abnormalities consistent with those seen in the *G4D* mouse model (septal defect, ECD, hypoplastic RV, and cardiomyopathy). 50 of these patients were obtained from the Children's Hospital Boston Cardiovascular Disease Registry. 33 patients with ECD were obtained from registries at Cincinnati Children's

Table 1
Non-synonymous *GATA4* mutations associated with congenital heart disease

Study	Study population	Nucleotide change	Amino acid change	Phenotype
Garg et al. 2003 [3]	Familial septal defects (2 families)	886G>A	G296S	ASD±VSD, PS (1 family; 1 case of ECD)
		1075delG	E359RfsX44	ASD (1 family)
Hiaryama-Yamada et al. 2005 [4]	Familial ASD (16 families)	1075delG	E359RfsX44	ASD (1 family)
		155C>T	S52F	ASD (1 family)
Okubo et al. 2004 [5]	Familial ASD (1 family)	1074delC	S358RfsX45	ASD±PS (1 family)
Sarkozy et al. 2005 [6]	ASD (16 families; 13 sporadic)	886G>A	G296S	ASD±PS (2 families)
Sarkozy et al.2005 [7]	ECD (9 families; 26 sporadic)	None	None	N/A
Nemer et al. 2006 [8]	Largely sporadic CHD (94 probands: 26 TOF; 30 VSD; 18 PS; 15 PDA; 12 ASD, 8 TA, 6 TGA, 5 CoA)	648C>G	E216D	TOF (2 sporadic cases)
Zhang et al. 2006 [9]	Largely sporadic CHD (99 probands: 36 VSD, 4 ASD, 11 TOF, ECD 1, 47 other)	None	None	N/A
Schluterman et al. 2007 [10]	Largely sporadic CHD (157 probands: 14 ASD, 18 VSD, 7 ECD, 18 TOF, 45 LVOTO, 55 other)	None	None	
This study	Largely sporadic CHD (237 probands; see Table 4)	487C>T	P163S	ECD (1 sporadic case)
		1037C>T	A346V	ECD (1 sporadic case)
		886G>T	296C	ASD+PS (1 family)
		1207C>A	L403M	Hypoplastic RV (1 sporadic case)

ASD, atrial septal defect; CoA, coarctation of the aorta; ECD, endocardial cushion defect; LVOTO, LV outflow tract obstruction; PS, pulmonary stenosis; PA, pulmonary atresia; PDA, patent ductus arteriosus; TA, tricuspid atresia; TGA, transposition of the great arteries; TOF, tetralogy of Fallot.

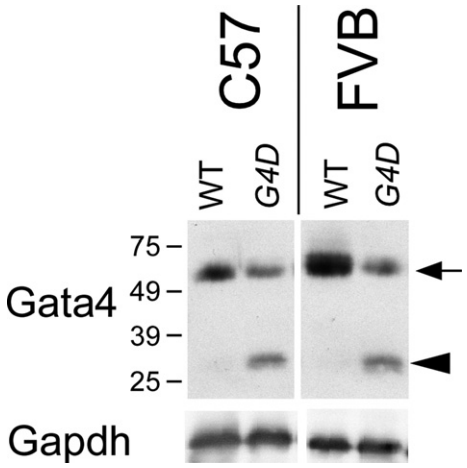

Fig. 1. Gata4 protein expression in WT and *G4D*fetal hearts. Gata4 protein in E14.5 WT and *G4D* hearts was measured by Western blotting. In *G4D* hearts, expression of full-length Gata4 protein (arrow) was reduced. A truncated protein lacking the N-terminal activation domain was expressed in *G4D* fetal hearts (arrowhead). Expression was not different in C57 and FVB strain backgrounds.

Hospital Medical Center and Children's Hospital of Philadelphia. Sequencing results for another 24 patients with cardiomyopathy were obtained via personal communications with M. Sarkar, C. Seidman, and J. Seidman. Genetic testing for specific molecular diagnoses was performed when clinically indicated. Individuals with known genetic abnormalities were excluded from this study. All studies were performed under protocols monitored by the respective Institutional Review Boards, and conformed with the principles outlined in the Declaration of Helsinki.

PCR-amplified genomic DNA was sequenced on both strands using primers designed to span the *GATA4* coding exons and exon/intron boundaries (Supplementary Table 1). 13 patients with ECDs were genotyped by denaturing high-performance liquid chromatography rather than by direct sequencing. Non-synonymous sequence variants were confirmed by re-sequencing or by allele-specific genotyping assays. Sequence positions are relative to *GATA4* cDNA (NM_002052) and protein (NP_002043). We predicted the functional effect of amino acid substitutions based on phylogenetic conservation and physiochemical properties of amino acid residues using the MAPP algorithm [14].

GATA4 was sequenced in 250 control individuals. In some cases, allele-specific genotyping assays were performed in additional controls. Some of these controls were previously reported [10].

3. Results

3.1. Murine model of Gata4 mutation

The mutant *Gata4* allele used in this study, $Gata4^{\Delta ex2}$, contains a deletion of the start codon and 46% of the coding region [11,15]. This allele does not express full-length protein. In fetal heart, but not in adult heart, the allele does express a truncated protein lacking the essential N-terminal transcriptional activation domain (Fig. 1) [15]. In vitro reporter assays showed loss of transcriptional activity and did not suggest dominant negative activity [15]. Prior in vivo characterization suggested that $Gata4^{\Delta ex2}$ is a loss of function allele [11,15,16]. although partial residual function or weak dominant negative activity cannot be excluded. We studied $Gata4^{\Delta ex2/WT}$ mice (abbreviated *G4D*), which express 50% reduced levels of full-length Gata4 protein (Fig. 1) [17].

We previously reported that in a mixed genetic background *G4D* hearts are structurally normal [11,17]. However, we found that *G4D* mice in an inbred C57 genetic background (*G4D*-C57) showed decreased survival (Fig. 2a). *G4D*-C57 mice were born at the expected Mendelian frequency, but suffered excess mortality in the perinatal period (52% mortality; Wilcoxon $P<0.0001$ vs. WT; Fig. 2b). Histological examination of a subset of the deceased *G4D*-C57 mice (20/56) demonstrated cardiac malformations in 85% (Fig. 2c). The distribution of malformations was similar to that observed in *G4D*-C57 fetuses (Table 2; see below). In some intriguing hearts, the long axis of the RV appeared divergent from the long axis of the LV (Fig. 2c). In addition to cardiac malformations, *G4D*-C57 mice have lung and diaphragm abnormalities, which may contribute to perinatal lethality [18].

3.2. Cardiac malformations in G4D-C57 mice

To systematically determine the spectrum of structural heart defects in *G4D*-C57 mice, we collected an unselected group of

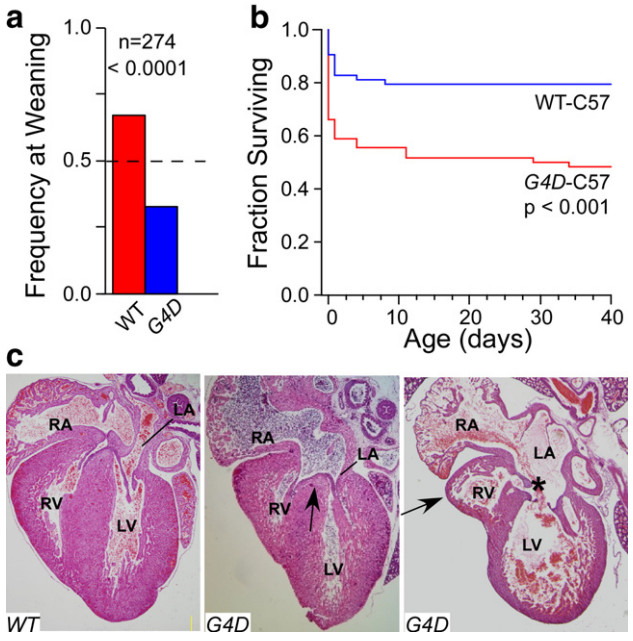

Fig. 2. Perinatal death of *Gata4*mutant mice. (a) Frequency of *G4D* or WT genotypes at weaning in the C57 strain background. The expected Mendelian frequency was 50% (dashed line). (b) Perinatal attrition of *G4D* mice in the C57 background. 141 births produced 56 *G4D* mice, of which 52% died, and 63 WT, of which 18% died. 22 pups were cannabilized as neonates and could not be genotyped. (c) Hearts from deceased WT and *G4D*-C57 neonates. The middle panel shows an ASD primum defect (arrow). The right panel shows a markedly hypoplastic RV. The RV apex (arrow) is distinct from the LV apex. There is also a CAVC defect (asterisk) that is mal-aligned so that it opens mostly into the LV.

Table 2
Cardiac malformations in unselected *G4D* late gestation embryos

Malformation	*G4D*-C57 (n=46)	*G4D*-FVB (n=23)	*G4D*-F1 (n=29)	*G4D*-back (n=172)
Normal	24% (11)	70% (16)*	88% (23)*	58% (99)*
ASD secundum	24% (11)		7% (2)	12% (20)
Isolated	4% (2)		7% (2)	10% (17)
With other defects	20% (9)			2% (3)
Endocardial cushion defect	59% (27)	4% (1)*	3% (1)*	24% (41)*
ASD primum	7% (3)			4% (7)
CAVC	38% (17)		3% (1)	13% (22)
Inlet VSD	18% (8)	4% (1)		7% (12)
Ventricles	26% (12)			
Membranous VSD	20% (9)	22% (5)		7% (10)
Muscular VSD	9% (4)	4% (1)		4% (6)
Small RV sinus	9% (4)	9% (2)		3% (5)

* $P<0.0001$, χ^2 test compared to *G4D*-C57.

late gestation embryos (post-coital days 15–19) and identified cardiac abnormalities on serial histological sections (Table 2). Out of 46 unselected late gestation *G4D*-C57 hearts examined, only 24% (11/46) appeared normal. Most malformations were severe (33/46, 72%), consisting of ECDs (27/46, 59%), VSDs (12/46, 26%), and hypoplasia of the RV (4/46, 9%). Isolated ASD secundum was observed in 2/46 (2%).

We observed a range of ECDs that included balanced complete atrioventricular canal (CAVC), LV-dominant CAVC, inlet VSD, and ASD primum (Figs. 3a–e). RV hypoplasia involved the inflow portion of the right ventricle and spared the outflow portion (Fig. 4). We observed 15 instances of severe RV hypoplasia; 5 occurred in the absence of ECD, and in 4 the apex of the RV was distinct from the apex of the LV (Fig. 2c). These findings show that heterozygous *Gata4* mutation causes aberrant endocardial cushion development and RV morphogenesis.

3.3. Influence of genetic background on G4D phenotype

Genetic background modifies the phenotypic expression of single gene mutations in mice and in humans [19–21]. To study the effect of genetic background on phenotypic expression of heterozygous *Gata4* mutation, we bred *G4D* into the pure FVB genetic background. In *G4D*-FVB fetuses, 15/21 (71%) hearts were normal (Table 2). The frequency of VSDs (5/23; 22%) and RV hypoplasia (2/23, 9%) was similar to that found in the C57 background. However, the frequency of ECDs was substantially lower (1/23, 4%; χ^2 $P<0.0001$). These data indicate that genetic modifiers increase the frequency of ECDs by 20-fold in *G4D*-C57 compared to *G4D*-FVB (59% in *G4D*-C57 vs. 3% in *G4D*-FVB).

To further characterize the strain-specific genetic modifiers, we crossed *G4D*-C57 mice to FVB obtain *G4D*-F1 mice. 88% of *G4D*-F1 hearts were normal, and only 1/33 (3%; χ^2 $P<0.0001$) had an ECD (Table 2). This was not due to differences between the $Gata4^{WT}$ allele in C57 versus FVB, because the strain contributing the wild-type allele did not influence survival (data not shown). These data suggest that genetic modifier(s) in the C57 strain that increase risk of ECD is (are) recessive.

To map the modifier(s), we crossed *G4D*-F1 mice to C57 to obtain *G4D*-backcross (*G4D*-back) embryos. We sectioned 172 such embryos in late gestation, and found cardiac malformations in 73 (42%; Table 2). ECDs occurred at the highest frequency (41/172, 24%), again consistent with recessive modifier(s) in C57. The ratio of males versus females was the same in affected versus unaffected embryos, suggesting that the modifier(s) were located on autosomes. We performed a whole genome linkage scan, genotyping 25 affected and 13 unaffected *G4D*-back embryos at 691 single-nucleotide polymorphisms (SNPs) informative between C57 and FVB. We did not identify any SNP where the C57 homozygous genotype was significantly enriched amongst affected mice (data not shown). The study had an 80% likelihood of identifying a suggestive linkage (LOD $\geq$ 2.46) between ECD and a single strong modifier (relative risk of 23). Thus, the absence of a significant association in this study suggests multiple modifiers, each with a lower relative risk.

3.4. Abnormal cardiac function in G4D mice

We previously reported that depressed ventricular function is a fully penetrant phenotype of *G4D* mice in a C57/FVB F1 genetic background [17]. We asked if ventricular function in adult *G4D* mice varied with strain background. Compared to WT, *G4D*-FVB mice had mild ventricular dysfunction while *G4D*-C57 mice exhibited moderate–severe ventricular dysfunc-

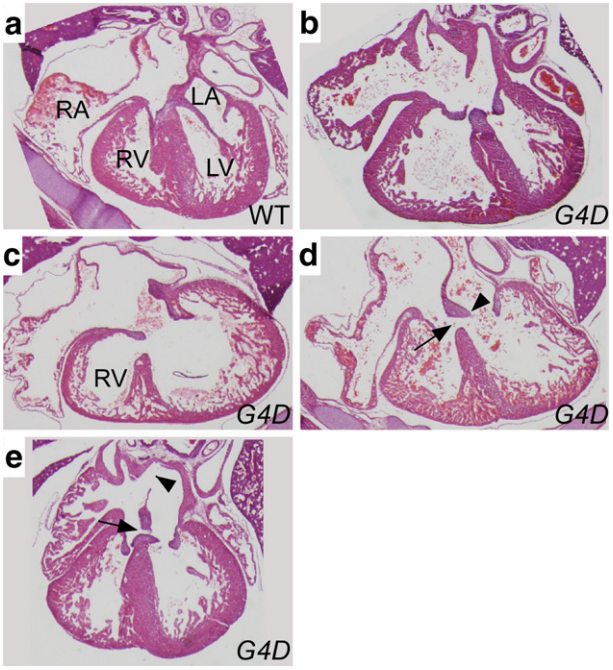

Fig. 3. ECDs in *G4D*-C57 late gestation embryos. (a–e) Hematoxylin and eosin-stained sections demonstrating a spectrum of ECDs. (a) WT control. (b) Well-balanced CAVC canal defect. (c) CAVC defect opening mainly into the left ventricle. The RV is moderately hypoplastic. (d) Inlet VSD. The atrial septum is intact, and there are two AV valves, albeit with highly primitive leaflets (arrowhead). The ventricular portion of the AV canal is not septated, resulting in an inlet VSD (arrow). (e) ASD primum (arrow) and ASD secundum (arrowhead). The ventricular portion of the AV canal is septated.

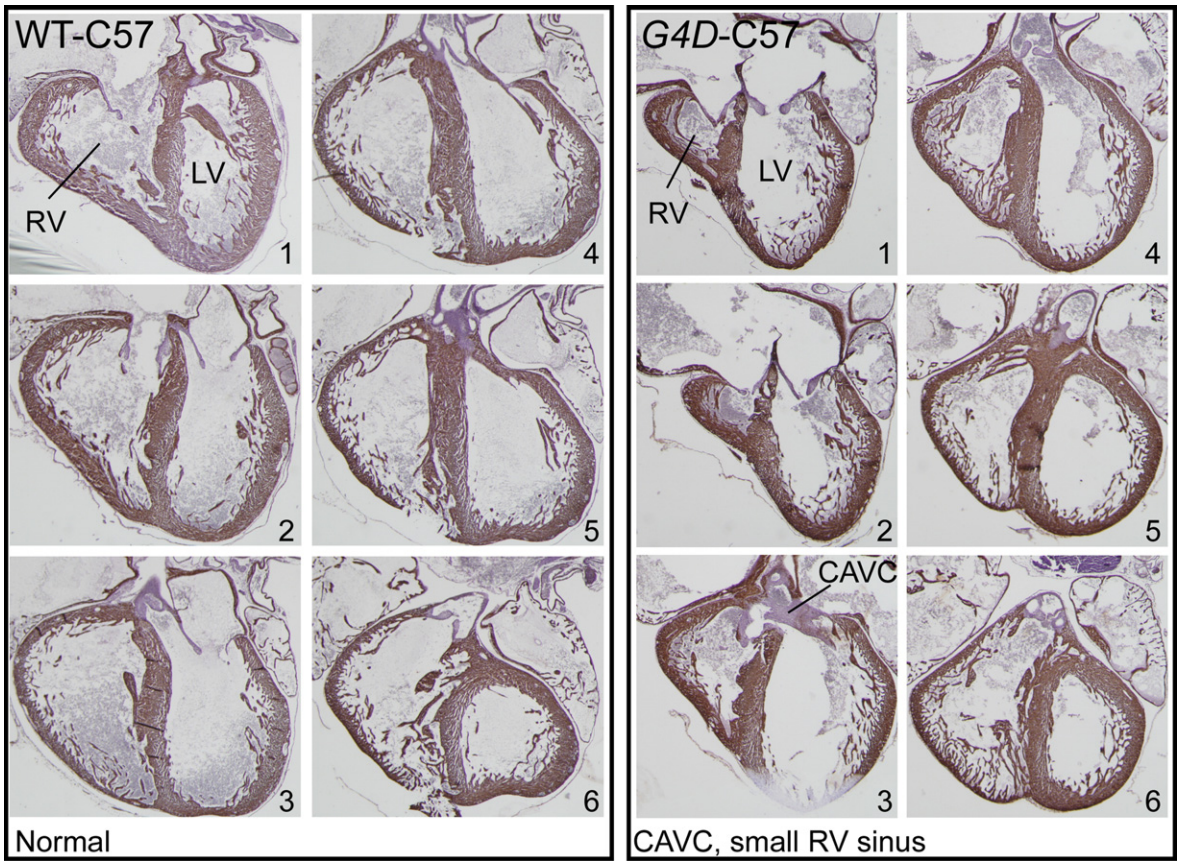

Fig. 4. RV hypoplasia in *G4D*-C57 late gestation embryo. Adjacent sections from an E18.5 WT-C57 (left box of six images) or *G4D*-C57 littermate (right box of six images). Myocardium was visualized by desmin immunostaining, and counterstained with hematoxylin. Numbers indicate order of sections. The hypoplastic inflow portion of the RV is shown in sections 1 and 2. The outflow portion of the RV was normal in size (sections 5 and 6). In this heart, RV hypoplasia occurred in association with CAVC (section 3).

tion (Table 3). This was not due to cardiac malformations because postmortem analysis included serial histological sectioning of each heart in this study. This showed only one ASD secundum in a *G4D*-C57 heart.

3.5. GATA4 mutations in human heart disease

To extend our study to human CHD, we analyzed the coding regions and splice donor/acceptor sites of *GATA4* in genomic DNA samples from 107 patients with cardiac abnormalities in the phenotypic spectrum of the *G4D* mouse model (septal defect, $n=8$; ECD, $n=43$; RV hypoplasia, in the context of DILV, $n=9$; or cardiomyopathy, $n=48$) (Table 4).

We identified several non-synonymous *GATA4* sequence variants. Those found in control only, or in probands and controls, are listed in Supplementary Table 2. Four non-synonymous *GATA4* sequence variants occurred in probands but not in controls (Table 5):

(1) G296C occurred in a proband with secundum ASD and pulmonary stenosis, and is similar to the previously described G296S mutation, which co-segregated with secundum ASDs and PS in two unrelated pedigrees (Table 1) [3,6]. The proband's father had the same G296C substitution and had a persistent LSVC to coronary sinus. A sibling also had a secundum ASD, but DNA was not available for genetic analysis. This sequence variant was not found in 500 control chromosomes (246 ethnically matched). G296 is invariant from *Xenopus* through human, and occurs in the DNA-binding domain. The related G296S mutation reduced GATA4 DNA-binding activity as well as binding to the transcription factor Tbx5 [3].

(2) L403M occurred in a proband with hypoplastic RV in the context of DILV. This patient also had a sinus venosus ASD. There was no family history of CHD. Parental DNA was not available for genotyping. This sequence variant was not found in 500 control chromosomes (62 ethnically matched). L403 is invariant from *Xenopus* through human, and occurs in the C-terminal domain that is required for transcriptional activation [22].

(3) and (4) P163S and A346V occurred in probands with ECDs. In each case, the family history was negative for CHD, but one parent was a carrier of the sequence variant. These carrier individuals did not have clinically apparent heart disease (direct echocardiographic studies were not available). These sequence variants were not found in 600 control chromosomes

Table 3
Gravimetric and echocardiographic assessment of ventricular function in *G4D* mice

	C57BL6/J		FVB/N	
	WT (n=7)	*G4D* (n=7)	WT (n=6)	*G4D* (n=5)
LVEDD (mm)	3.50±0.06	3.92±0.23	3.52±0.06	3.64±0.10
LVESD (mm)	1.72±0.12	2.65±0.23[a]	1.54±0.09	1.93±0.05[a,c]
PWth (mm)	0.49±0.02	0.57±0.06	0.52±0.03	0.50±0.02
FS (%)	51±1	33±2[a]	56±2	47±2[b,c]
HR (bpm)	680±17	639±46	727±8	733±26
BW (g)	30.0±0.9	30.3±0.5	29.1±0.7	28.2±1.7
TL (mm)	17.1±0.1	17.1±0.1	171±0.1	17.2±0.1
HW (mg)	158±7	167±8	133±4	134±8[c]
LuW (mg)	148±9	148±7	145±8	137±6
HW/BW (mg/g)	5.3±0.2	5.5±0.3	4.6±0.1	4.8±0.1
HW/TL (mg/mm)	9.2±0.4	9.8±0.5	7.8±0.3	7.8±0.5[c]

Results are shown as mean±SEM. Groups were compared by ANOVA with Tukey–Kramer's HSD post-hoc test.
LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; PWth, posterior wall thickness; FS, fractional shortening; HR, heart rate; bpm, beats per minute; BW, body weight; TL, tibial length; HW, heart weight; LuW, lung weight.
[a] $p<0.01$ compared to WT control of the same strain.
[b] $p<0.05$ compared to WT control of the same strain.
[c] $p<0.05$ for *G4D*-C57 compared to *G4D*-FVB.

(346 ethnically matched). The proline at position 163 is invariant from *Xenopus* through human, and occurs in a transactivation domain that is required for GATA4 activity and is conserved in GATA4, GATA5, and GATA6 [22]. The residue at position 346 is either alanine or serine (a conservative substitution in the BLOSUM62 matrix [23]) in *Xenopus* through human. This residue occurs in the C-terminal domain required for transcriptional activation [22]. The A346V substitution is non-conservative in the BLOSUM62 matrix [23].

We modeled the effect of these four substitutions on protein function using the MAPP algorithm [14]. Each of the four substitutions was predicted to be deleterious to protein function.

Although cardiomyopathy was a highly penetrant phenotype in *G4D* mice, we did not find *GATA4* mutations among 48 patients with cardiomyopathy (Table 4). Conversely, none of the 22 patients with *GATA4* sequence alteration reported in this study or a prior study [3] had ventricular dysfunction attributable to *GATA4* mutation. These data suggest that heterozygous *GATA4* mutation is not a frequent cause of cardiomyopathy in humans.

4. Discussion

4.1. Spectrum of phenotypes associated with murine Gata4 mutation

Using tissue-restricted gene inactivation approaches, we previously showed that *Gata4* is required in the myocardial compartment for normal myocardial growth and RV morphogenesis [24], and in endocardially derived structures for normal atrioventricular valve development [15]. These processes were also disrupted by a hypomorphic mutation of *Gata4*, which reduced Gata4 protein by 70% and resulted in embryonic lethality due to defects in myocardial growth, endocardial cushion development, and outflow tract alignment. In this work, we extend these findings to show for the first time that heterozygous mutation of *Gata4* in mice is sufficient to disrupt myocardial growth and endocardial cushion development. We did not observe conotruncal abnormalities or abnormalities of outflow tract alignment in heterozygous *Gata4* mutant mice, suggesting that these abnormalities occur only after a greater perturbation of *Gata4* activity. Collectively, these findings emphasize the importance of maintaining precise levels of *GATA4* activity for normal development of these structures. Reduction of *GATA4* activity through genetic mutation of *GATA4* or interacting factors, or environmental influences (e.g., retinoic acid deficiency [25]), might lead to a similar spectrum of cardiac phenotypes.

Although human mutation of one copy of *GATA4* causes familial septal defects, mice with mutation of one copy of *Gata4* were previously reported to be normal [26–28]. This apparent difference between mice and humans appeared to prohibit using the heterozygous mutant mouse model to gain insights into the spectrum of abnormalities that might occur in humans with heterozygous *GATA4* mutation. However, by studying the phenotype of heterozygous *Gata4* mutant mice in different inbred strain backgrounds, we show that murine mutation of one copy of *Gata4* is sufficient to lead to septal defect, ECD, RV hypoplasia, and cardiomyopathy, albeit with reduced penetrance and variable expressivity.

4.2. Modifiers of cardiovascular phenotype due to murine Gata4 mutation

While reduced penetrance and variable expressivity have been frequently observed in studies of single gene mutations associated with CHD [19–21,29], the cause of this variability is not well understood. In the case of heterozygous *Gata4* mutation in mice, we show that genetic modifiers strongly influence phenotypic expression, as the C57 background increased the frequency of ECDs by 20-fold over the FVB background. LV dysfunction was also more severe in the C57 compared to the FVB background. Our screen for genetic

Table 4
Patient characteristics

Cardiac lesion	Patients, # (%)	*GATA4* alteration[a], # (%)	Probands with family history
Endocardial cushion defects	42 (39)	2 (4.8)	0
Double inlet LV	9 (8)	1 (11.1)	0
ASD/VSD	8 (7)	1 (12.5)	1
Cardiomyopathy[b]	48 (45)	0 (0)	6
Total	107	4 (3.7)	

[a] GATA4 alteration is defined as a non-synonymous sequence alteration not found in control individuals.
[b] 24 of these patients were via personal communications from M. Sarkar, C. Seidman, and J. Seidman.

Table 5
Non-synonymous *GATA4* sequence variants associated with CHD found in this study

Proband	Sequence variant	AA change	Conservation	Affected domain	Controls		Cardiac phenotype	Note
					All, # (est allelic freq)	Ethnically matched, # (est allelic freq)		
1	1207C>A	L403M	XMRPH	C-Terminal domain	0/500 (0–0.007)	0/62 (0–0.058)	Hypoplastic RV in the context of [S,D,D] DILV with sinus venosus atrial septal defect	
2	886G>T	G296C	XMRPH	C-Terminal Zn finger/ NLS junction	0/500 (0–0.007)	0/246 (0–0.015)	Secundum ASD, valvar PS	Sister: ASD 2, geno unknown; Father: LSVC to CS, G296C.
3	487C>T	P163S	XMRPH	TAD2	0/600 (0–0.006)	0/346 (0–0.011)	ECD (Primum ASD, cleft MV)	Father P163S reportedly unaffected.
4	1037C>T	A346V	MPH (Ser in XR)	C-Terminal domain	0/600 (0–0.006)	0/346 (0–0.011)	ECD (Primum ASD, cleft MV)	Mother A346V reportedly unaffected.

Proband 1 was Libyan Jewish. Probands 2–4 were Caucasian. "Conservation" indicates identical residue in *Xenopus laevis* (X), *Mus musculus* (M), *Rattus norvegicus* (R), *Sus scrofa* (pig, P), and *Homo sapiens* (H). "Controls" indicates the number of control alleles with the sequence variant, and the number of control alleles examined. This is reported for all controls and for ethnically matched controls. "Est allelic freq" indicates the 95% confidence estimate of the allelic frequency in controls. The upper bound of this estimate was calculated from observing zero sequence variants in *n* control chromosomes, using a binomial distribution [32]. ASD 2, secundum ASD; LSVC, left superior vena cava; CS, coronary sinus; NLS, nuclear localization sequence; MV, mitral valve; TAD2, second N-terminal transcriptional activation domain.

modifiers of the ECD phenotype did not identify a single strong genetic modifier, suggesting that two or more weaker modifiers are responsible.

An inference from our experiments is that epigenetic factors must also have an important influence on the expression of cardiovascular phenotypes. Despite extensive inbreeding (now backcrossed 13 generations), *G4D* mutant mice display considerable phenotypic variation and partial penetrance that cannot be accounted for by genetic factors. The controlled breeding environment argues against a significant contribution of environmental factors to the observed phenotypic variation. By exclusion, this suggests that stochastic events contribute to the phenotypic heterogeneity. Similar phenotypic heterogeneity was previously noted in a careful study of *Hey2* null mice in highly inbred strain backgrounds [29], indicating that the important role of epigenetic factors in modulating phenotypic expression extends beyond *Gata4* mutations.

4.3. Human GATA4 mutations

In a panel of 107 patients with largely sporadic CHD and cardiac phenotypes that overlapped those observed in the *G4D* mouse model, we found four *GATA4* non-synonymous sequence variants (G296C, L403M, P163S, and A346V) that did not occur in control individuals. These are likely disease-causing mutations, as each is a sequence variant that alters a highly conserved residue and that was not found in controls. A strength of our study was the large number of control chromosomes analyzed (500–600 total; 62–346 ethnically matched; Table 5). Computation modeling suggested that each substitution was deleterious to protein function. Further, the related G296S mutation has been previously reported to be disease causing [3,6]. One parent of each of the probands with P163S and A346V mutation carried the mutation but did not have overt clinical disease. This likely reflects reduced penetrance, which might be expected based on the mouse model.

We found *GATA4* mutations in two patients with ECD. An additional patient with ECD and *GATA4* mutation has previously been reported as a member of an extended pedigree with *GATA4* mutation and predominantly ASD or VSD [3]. These data indicate that *GATA4* mutation can cause ECD in humans as well as in mice. The estimated frequency of *GATA4* mutation among sporadic ECD cases (2/43 in this study plus 0/34 from the literature (Table 1)=2.6%) is significant for a single gene.

No single gene mutation has been previously associated with DILV in humans. This cardiac malformation has been proposed to result from severe RV hypoplasia with consequent mal-positioning of the atrioventricular septum [30], or from abnormal endocardial cushion development [31]. Since *GATA4* is a crucial regulator of both RV chamber morphogenesis and endocardial cushion development [15,24], an association of DILV with *GATA4* mutation is consistent with its known roles in heart development. In *G4D* mice, we observed two malformations that may represent *forme frustes* of DILV: mal-aligned CAVC in which the common AV valve opened predominantly into the LV (Fig. 3c), and severe hypoplasia of the RV sinus (Fig. 4).

4.4. Use of heterozygous mouse models to model human disease

While constitutive and conditional knockout studies illuminate the essential function of genes, the complete ablation of gene activity in these models is often not representative of human disease. In many cases, heterozygous mouse mutants more closely model the perturbations in gene activity that occur in human disease as a result of heterozygous mutation or environmental influences. In this study, we carefully examined the phenotype of mice with heterozygous *Gata4* mutation, in order to generate hypotheses regarding potential cardiac phenotypes that may be associated with *GATA4* mutation in humans. Of the 107 patients in our study with cardiac pheno-

types overlapping those seen in the mouse model, 4 patients had *GATA4* mutation. In contrast, we did not find *GATA4* mutation in 126 patients with cardiac phenotypes not seen in the mouse model (conotruncal anomalies, $n=34$; heterotaxy, $n=7$; Ebstein's anomaly, $n=5$; left-sided obstruction, $n=81$; data not shown). Out of the five types of phenotypes that were present in the mouse model (ECD, RV hypoplasia, ASD, VSD, cardiomyopathy), four types are associated with *GATA4* mutation in humans (ECD, RV hypoplasia in the context of DILV, ASD, VSD). These results suggest that careful study of heterozygous mutant mouse models is a productive experimental strategy for developing hypotheses regarding potential human phenotypes.

Given the high penetrance of cardiomyopathy in the *G4D* mouse model, it was notable that we did not find *GATA4* mutations among patients with cardiomyopathy. Moreover, 22 patients with *GATA4* mutation did not exhibit cardiomyopathy. This might reflect a difference between the specific mutations studied in mice and humans, or a difference in phenotypic expression between mouse and humans. Thus, while mouse models are useful for generation of hypotheses regarding human phenotypes, the hypotheses require testing in humans.

In this study, we showed that heterozygous *Gata4* mutation in a mouse model caused ECD, RV hypoplasia, septal defects, and cardiomyopathy. Among patients with CHD, we found *GATA4* mutations associated with overlapping cardiac phenotypes, namely ECD, RV hypoplasia in the context of DILV, and septal defects. These data support the importance of fine regulation of *GATA4*-dependent pathways in the development of these structures, and identify *GATA4* as a cause of sporadic ECD and DILV in humans. Additional targeted studies of more patients with ECD and particularly RV hypoplasia are needed to determine the degree to which *GATA4* mutation contributes to these forms of CHD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yjmcc.2007.06.004.

References

[1] Srivastava D, Olson EN. A genetic blueprint for cardiac development. Nature 2000;407:221–6.
[2] Ransom J, Srivastava D. The genetics of cardiac birth defects. Semin Cell Dev Biol 2007;18:132–9.
[3] Garg V, Kathiriya IS, Barnes R, Schluterman MK, King IN, Butler CA, et al. *GATA4* mutations cause human congenital heart defects and reveal an interaction with TBX5. Nature 2003;424:443–7.
[4] Hirayam-Yamada K, Kamisago M, Akimoto K, Aotsuka H, Nakamura Y, Tomita H, et al. Phenotypes with *GATA4* or *NKX2.5* mutations in familial atrial septal defect. Am J Med Genet, A 2005;135:47–52.
[5] Okubo A, Miyoshi O, Baba K, Takagi M, Tsukamoto K, Kinoshita A, et al. A novel *GATA4* mutation completely segregated with atrial septal defect in a large Japanese family. J Med Genet 2004;41:e97.
[6] Sarkozy A, Conti E, Neri C, D'Agostino R, Digilio MC, Esposito G, et al. Spectrum of atrial septal defects associated with mutations of NKX2.5 and GATA4 transcription factors. J Med Genet 2005;42:e16.
[7] Sarkozy A, Esposito G, Conti E, Digilio MC, Marino B, Calabro R, et al. *CRELD1* and *GATA4* gene analysis in patients with nonsyndromic atrioventricular canal defects. [Letter]Am J Med Genet, A 2005;139(3): 236–8.
[8] Nemer G, Fadlalah F, Usta J, Nemer M, Dbaibo G, Obeid M, et al. A novel mutation in the *GATA4* gene in patients with tetralogy of Fallot. Hum Mutat 2006;27:293–4.
[9] Zhang L, Tumer Z, Jacobsen JR, Andersen PS, Tommerup N, Larsen LA. Screening of 99 Danish patients with congenital heart disease for *GATA4* mutations. Genet Test 2006;10:277–80.
[10] Schluterman MK, Krysiak AE, Kathiriya IS, Abate N, Chandalia M, Srivastava D, et al. Screening and biochemical analysis of *GATA4* sequence variations identified in patients with congenital heart disease. Am J MedGenet, A 2007;143A:817–23.
[11] Pu WT, Ishiwata T, Juraszek AL, Ma Q, Izumo S. *GATA4* is a dosage-sensitive regulator of cardiac morphogenesis. Dev Biol 2004;275:235–44.
[12] Xu S, Atchley WR. Mapping quantitative trait loci for complex binary diseases using line crosses. Genetics 1996;143:1417–24.
[13] Broman KW, Wu H, Sen S, Churchill GA. R/qtl: QTL mapping in experimental crosses. Bioinformatics 2003;19:889–90.
[14] Stone EA, Sidow A. Physicochemical constraint violation by missense substitutions mediates impairment of protein function and disease severity. Genome Res 2005;15:978–86.
[15] Rivera-Feliciano J, Lee KH, Kong SW, Rajagopal S, Ma Q, Springer Z, et al. Development of heart valves requires *Gata4* expression in endothelial-derived cells. Development 2006;133:3607–18.
[16] Bosse T, Piaseckyj CM, Burghard E, Fialkovich JJ, Rajagopal S, Pu WT, et al. *Gata4* is essential for the maintenance of jejunal–ileal identities in the adult mouse small intestine. Mol Cell Biol 2006;26:9060–70.
[17] Bisping E, Ikeda S, Kong SW, Tarnavski O, Bodyak N, McMullen JR, et al. *Gata4* is required for maintenance of postnatal cardiac function and protection from pressure overload-induced heart failure. Proc Natl Acad Sci U S A 2006;103:14471–6.
[18] Jay PY, Bielinska M, Erlich JM, Mannisto S, Pu WT, Heikinheimo M, et al. Impaired mesenchymal cell function in *Gata4* mutant mice leads to diaphragmatic hernias and primary lung defects. Dev Biol 2007;301: 602–14.
[19] Benson DW, Silberbach GM, Kavanaugh-McHugh A, Cottrill C, Zhang Y, Riggs S, et al. Mutations in the cardiac transcription factor NKX2.5 affect diverse cardiac developmental pathways. J Clin Invest 1999;104:1567–73.
[20] Goldmuntz E, Geiger E, Benson DW. NKX2.5 mutations in patients with tetralogy of Fallot. Circulation 2001;104:2565–8.
[21] McElhinney DB, Geiger E, Blinder J, Benson DW, Goldmuntz E. NKX2.5 mutations in patients with congenital heart disease. J Am Coll Cardiol 2003;42:1650–5.
[22] Morrisey EE, Ip HS, Tang Z, Parmacek MS. GATA-4 activates transcription via two novel domains that are conserved within the GATA-4/5/6 subfamily. J Biol Chem 1997;272:8515–24.
[23] Henikoff S, Henikoff JG. Amino acid substitution matrices from protein blocks. Proc Natl Acad Sci U S A 1992;89:10915–9.
[24] Zeisberg EM, Ma Q, Juraszek AL, Moses K, Schwartz RJ, Izumo S, et al. Morphogenesis of the right ventricle requires myocardial expression of *Gata4*. J Clin Invest 2005;115:1522–31.
[25] Ghatpande S, Ghatpande A, Zile M, Evans T. Anterior endoderm is sufficient to rescue foregut apoptosis and heart tube morphogenesis in an embryo lacking retinoic acid. Dev Biol 2000;219:59–70.

[26] Molkentin JD, Lin Q, Duncan SA, Olson EN. Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. Genes Dev 1997;11:1061–72.
[27] Kuo CT, Morrisey EE, Anandappa R, Sigrist K, Lu MM, Parmacek MS, et al. GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. Genes Dev 1997;11:1048–60.
[28] Xin M, Davis CA, Molkentin JD, Lien CL, Duncan SA, Richardson JA. A threshold of GATA4 and GATA6 expression is required for cardiovascular development. Proc Natl Acad Sci U S A 2006;103: 11189–94.
[29] Sakata Y, Koibuchi N, Xiang F, Youngblood JM, Kamei CN, Chin MT. The spectrum of cardiovascular anomalies in CHF1/Hey2 deficient mice reveals roles in endocardial cushion, myocardial and vascular maturation. J Mol Cell Cardiol 2006;40:267–73.
[30] van Praagh R, Plett JA, van Praagh S. Single ventricle. Pathology, embryology, terminology and classification. Herz 1979;4:113–50.
[31] Jiao K, Langworthy M, Batts L, Brown CB, Moses HL, Baldwin HS. Tgf {beta} signaling is required for atrioventricular cushion mesenchyme remodeling during in vivo cardiac development. Development 2006;133: 4585–93.
[32] Clopper CJ, Pearson ES. The use of confidence or fiducial limits illustrated in the case of the binomial. Biometrika 1934;26:404–13.