Heterogeneity of Genetic Modifiers Ensures Normal Cardiac Development

Julia B. Winston, MS; Jonathan M. Erlich, BS; Courtney A. Green, BS; Ashley Aluko; Kristine A. Kaiser, MD; Mai Takematsu, MD; Robert S. Barlow, MD, PhD; Ashish O. Sureka, MD; Martin J. LaPage, MD; Luc L. Janss, PhD; Patrick Y. Jay, MD, PhD

Background—Mutations of the transcription factor Nkx2-5 cause pleiotropic heart defects with incomplete penetrance. This variability suggests that additional factors can affect or prevent the mutant phenotype. We assess here the role of genetic modifiers and their interactions.

Methods and Results—Heterozygous Nkx2-5 knockout mice in the inbred strain background C57Bl/6 frequently have atrial and ventricular septal defects. The incidences are substantially reduced in the Nkx2-5+/− progeny of first-generation (F1) crosses to the strains FVB/N or A/J. Defects recur in the second generation (F2) of the F1×F1 intercross or backcrosses to the parental strains. Analysis of >3000 Nkx2-5+/− hearts from 5 F2 crosses demonstrates the profound influence of genetic modifiers on disease presentation. On the basis of their incidences and coincidences, anatomically distinct malformations have shared and unique modifiers. All 3 strains carry susceptibility alleles at different loci for atrial and ventricular septal defects. Relative to the other 2 strains, A/J carries polymorphisms that confer greater susceptibility to atrial septal defect and atrioventricular septal defects and C57Bl/6 to muscular ventricular septal defects. Segregation analyses reveal that ≥2 loci influence membranous ventricular septal defect susceptibility, whereas ≥2 loci and at least 1 epistatic interaction affect muscular ventricular and atrial septal defects.

Conclusions—Alleles of modifier genes can either buffer perturbations on cardiac development or direct the manifestation of a defect. In a genetically heterogeneous population, the predominant effect of modifier genes is health.

Key Words: genetic variation ■ genetics ■ heart defects, congenital

Significant progress has been made toward defining genetic origins in congenital heart disease.1,2 Current knowledge of the few dozen genes implicated in cardiac development, however, does not explain the basis of common epidemiological and clinical observations. The incidence of heart defects in newborns is 0.5% to 1%, making it a leading cause of death in children. Still, the vast majority are born with a normal heart. Selection against fetuses that have cardiac defects does not cause the prevalence of the norm.3,4 Insults on the embryonic heart may be rare or weak, or mechanisms may have evolved to ensure the robustness of development. Direct evidence for either hypothesis is minimal, but the latter can better explain the breadth of observations related to incomplete penetrance and phenotypic variability.5,6

Editorial see p 1277
Clinical Perspective on p 1321

Although family studies led to the discovery of mutations of prototypical cardiac developmental genes such as TBX5, Nkx2-5, and GATA4,7–10 most cases of congenital heart disease have no identified cause or association. Such sporadic cases may be due to an unknown teratogen, spontaneous mutation, or inconsistent expression of an inherited origin. The last possibility is supported by the increased incidence of congenital heart disease in subsequent first-degree relatives, 2% to 11%, compared with the general population.11–13 Some normal relatives of sporadic patients must somehow escape the manifestation of a genetic mutation. Furthermore, among the affected relatives, the cardiac phenotypes are usually dissimilar and of wide-ranging severity.11,13 Modifying factors likely influence the incomplete penetrance and phenotypic variability associated with the expression of a causative mutation. In a family, the activity of modifiers in pathways involved in the development of an affected cardiac structure could determine whether a particular relative develops a heart defect, its type, and its severity. The unknown factors could be genetic, environmental, or entirely stochastic. Of these, genetic modifiers are most amenable to investigation in a laboratory where environmental factors can be controlled between experimental groups.
Small studies have demonstrated the effect of the background strain on cardiac phenotypes related to embryonic viability and myocardial, valvar, or ventricular septal morphology in Tbx5, Gata4, and Hey2 mutations. The extent to which genetic modifiers contribute to incomplete penetrance and phenotypic variability as observed in humans might only be appreciated, however, in a much larger study. In this regard, the diverse anatomic phenotypes described in knockout animals in the inbred mouse strain C57Bl/6 and in mouse strains and crosses to the strains FVB/N and A/J. A survey of thousands of generations. The knockout allele was generated and genotyped as described. At birth (P0) or at weaning (P21). Pups were collected within hours of birth or at weaning.

**Table. Observed and Expected Distributions of Nkx2-5+/− and Wild-Type Pups**

<table>
<thead>
<tr>
<th>Background</th>
<th>Nkx2-5 Genotype</th>
<th>Age</th>
<th>n</th>
<th>Observed</th>
<th>Expected</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57Bl/6</td>
<td>HET×HET</td>
<td>P21</td>
<td>505</td>
<td>62.5</td>
<td>66.7</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>C57Bl/6</td>
<td>HET×WT</td>
<td>P21</td>
<td>854</td>
<td>45.3</td>
<td>50</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>C57Bl/6</td>
<td>HET×WT</td>
<td>P0</td>
<td>100</td>
<td>56</td>
<td>50</td>
<td>NS</td>
</tr>
<tr>
<td>C57Bl/6×FVB/N F1</td>
<td>HET×WT</td>
<td>P21</td>
<td>214</td>
<td>46.7</td>
<td>50</td>
<td>NS</td>
</tr>
<tr>
<td>C57Bl/6×A/J F1</td>
<td>HET×WT</td>
<td>P21</td>
<td>156</td>
<td>51.9</td>
<td>50</td>
<td>NS</td>
</tr>
<tr>
<td>C57Bl/6×FVB/N F2</td>
<td>HET×HET</td>
<td>P21</td>
<td>212</td>
<td>66.5</td>
<td>66.7</td>
<td>NS</td>
</tr>
<tr>
<td>C57Bl/6×A/J F2</td>
<td>HET×HET</td>
<td>P0</td>
<td>562</td>
<td>64.4</td>
<td>66.7</td>
<td>NS</td>
</tr>
</tbody>
</table>

Shown are the observed and expected distributions of Nkx2-5+/− and wild-type pups in various crosses either at birth (P0) or at weaning. The experiments were approved by the animal studies committee at Washington University School of Medicine.

**Diagnosis of Congenital Heart Defects**

Neonatal pups were collected each morning within hours of birth to prevent cannibalization of animals that have serious congenital heart defects. The pups were euthanized. The thorax was fixed in 10% neutral buffered formalin overnight and then transferred to 70% ethanol. Fixed hearts were dissected, embedded in paraffin, and entirely sectioned in the frontal plane at 6-μm thickness. Each heart was inspected under a 5× microscope objective by at least 2 individuals. Defects were diagnosed by the appearance of the septae, valves, or blood in consecutive sections. Genomic DNA was isolated from every animal by phenol-chloroform extraction.

**Statistical Analyses**

To compare the genotypic distribution in a particular strain or cross with the expected mendelian ratio, all the pups from consecutive litters were counted up to at least 100 animals. Statistically significant deviation was defined in a χ² test by P<0.05.

The incidences of specific types of heart defects in crosses were compared by a 2-tailed Fisher exact test. The coincidence of heart defects in the same mouse was evaluated by comparison with the expected incidence, as determined by the product of their separate incidences, through the use of a χ² test.

Correlation of muscular and membranous ventricular septal defect (VSD) incidence with the average number of C57Bl/6 alleles per locus as expected from the mendelian distribution in the F2 crosses was evaluated by the Pearson product-moment correlation, followed by a 2-tailed t test with significance defined by P<0.05.

Segregation models were analyzed by comparison of their predictions and observed incidences by a χ² test. The models and derivation of equations are detailed in the Materials section in the online-only Data Supplement.

**Results**

**Newborn Nkx2-5+/− Animals in the C57Bl/6 Background Have a High Incidence of ASDs and VSDs**

Human Nkx2-5 mutations cause congenital heart defects, some of which are lethal if untreated in the newborn period. Although such serious defects in Nkx2-5+/− mice have not been described, we observed that ≈17% of Nkx2-5+/− pups in the C57Bl/6 background were missing at 2 to 3 weeks of age when they were weaned from their mothers. The attrition of Nkx2-5+/− animals occurred after birth because newborn pups were obtained at the expected mendelian ratio (the Table). We therefore examined newborn hearts that were...
collected within hours of birth. A high incidence of ASD of the secundum type was found in Nkx2-5/H11001/H11002 but not wild-type hearts (Figures 1 and 2). A secundum ASD was diagnosed by an insufficiency in covering the fossa ovale caused by a deficiency in its rim or of the septum primum. Hearts in which a potentially small ASD could not be distinguished from a patent foramen ovale were considered normal. The ASD incidence is greater than the 20% previously reported by 2 different groups for adult Nkx2-5/H11001/H11002 animals in the C57Bl/6 background possibly because of spontaneous closure or postnatal death.20–22

VSDs of the membranous and muscular types were also found in Nkx2-5/H11001/H11002 but not wild-type hearts (Figures 1 and 2). VSDs were not previously reported in adult animals possibly because of spontaneous closure or death from pulmonary overcirculation in the newborn period, as seen in mouse mutants that have a persistent patent ductus arteriosus.23

Previous investigators have detected aortic stenosis by Doppler echocardiography in live animals and bicuspid aortic valves by gross examination of adult Nkx2-5/+ animals.21 Given that we examined histological sections, minor valvular abnormalities that might cause regurgitation or stenosis were difficult to assess. Abnormal blood flow patterns have also been described by fetal echocardiography in association with severe lesions like truncus arteriosus.24 Severe defects and ones not easily detected by echocardiography like small VSDs resulting from physiologically elevated right-sided pressures in the fetus and newborn are easily recognized because of the greater spatial resolution of microscopy.

Nkx2-5/+ Animals in Hybrid Strain Backgrounds Generally Have Normal Cardiac Anatomy

Nkx2-5+/+ animals in the C57Bl/6 background were outcrossed to the FVB/N strain. The F1 progeny, which are C57Bl/6×FVB/N hybrids, showed the expected mendelian ratio of Nkx2-5+/+ pups at weaning (the Table). The absence of neonatal lethal defects was confirmed by direct inspection. Only 1 small VSD and 6 ASDs were found among 80 Nkx2-5+/+ F1 newborn mouse hearts. All 52 wild-type hearts were normal. The incidence of either septal defect in the Nkx2-5+/+ C57Bl/6×FVB/N F1 hybrids is significantly lower compared with the C57Bl/6 background (Figure 2).

Nkx2-5+/+ animals from the C57Bl/6×AJ F1 hybrid cross similarly showed the expected mendelian distribution of genotypes at weaning (the Table). Direct inspection confirmed a reduced incidence of heart defects compared with the C57Bl/6 background, none of which was expected to be

Figure 1. ASDs and VSDs in newborn Nkx2-5+/+ animals. An intact ventricular septum (A) and a normal atrial septum with a patent foramen ovale (B) in wild-type (WT) hearts. Membranous VSD (C) and muscular VSD (D) in Nkx2-5+/+ hearts. E, Secundum ASD in an Nkx2-5+/+ heart. Arrowheads point to the relevant defect in each section. Ao indicates aorta; RA, right atrium; LA, left atrium; RV, right ventricle; and LV, left ventricle.

Figure 2. The incidence of septal defects in wild-type (WT) and Nkx2-5+/+ animals in the C57Bl/6 background and in F1 crosses to FVB/N and A/J. The incidence of ASDs and VSDs in Nkx2-5+/+ hearts is substantially reduced in the F1 hybrids compared with the C57Bl/6 parental strain (P<0.001).
lethal. No VSDs and only 4 ASDs were found among 54 C57Bl/6/H11003 A/J F1 Nkx2-5/H11001/H11002 newborn hearts. All 48 wild-type hearts were normal (Figure 2).

The C57Bl/6×FVB/N F2 Crosses Reveal ASD and VSD Susceptibility Alleles in Both Strains

The C57Bl/6×FVB/N F1 hybrid data suggest that C57Bl/6 carries ≥1 recessive alleles of modifier genes that increase susceptibility to heart defects in the presence of Nkx2-5 mutation. The data do not exclude the possibility of recessive susceptibility alleles from FVB/N at other loci. To investigate these possibilities, Nkx2-5/H11001/H11002 F1 animals were backcrossed to C57Bl/6 and FVB/N or intercrossed to generate F2 progeny. Newborn F2 pups showed the expected mendelian distribution of genotypes, which indicates the absence of selection against defects that could cause prenatal demise (the Table).

VSD and ASD were more common in the Nkx2-5/H11001/H11002 animals from each of the C57Bl/6×FVB/N F2 crosses than from the F1 but less common than in C57Bl/6 (P<0.001 for comparisons with both the F1 and C57Bl/6). A VSD was present in 12% to 16% of the Nkx2-5/H11001/H11002 F2 hearts, and an ASD was present in 4% to 7% (Figure 3). These results indicate that at least 2 loci affect VSD and ASD susceptibility in the presence of Nkx2-5 mutation. C57Bl/6 and FVB/N carry recessive susceptibility alleles at different loci, given the increased incidence in the parental backcrosses compared
with the F1. The Nkx2-5+/− F2 may have a lower incidence of particular defects compared with C57Bl/6 either because FVB/N susceptibility genotypes have smaller effects or because interactions between C57Bl/6 and FVB/N alleles reduce risk.

The C57Bl/6 × A/J F2 Crosses Reveal A/J Susceptibility Alleles for ASD and Atrioventricular Septal Defects

Similar to the C57Bl/6 × FVB/N F2 results, the Nkx2-5+/− F2 progeny from the C57Bl/6 × A/J intercross and C57Bl/6 backcross showed an incidence of VSD and ASD greater than the F1 and less than C57Bl/6 (P < 0.001 for each of the comparisons; Figure 3). The backcross to A/J was not performed. Comparing the C57Bl/6 × A/J with the C57Bl/6 × FVB/N F2 crosses revealed 2 significant differences.

First, ASDs occurred at a higher frequency in the C57Bl/6 × A/J F2 intercross than in the C57Bl/6 × FVB/N (P < 0.001). There was a similar trend between the F2 backcrosses to C57Bl/6 that was not significant possibly because of the smaller sample sizes (P < 0.01). A/J may carry polymorphisms that confer greater susceptibility specifically to ASDs in the presence of Nkx2-5 mutation. The A/J polymorphisms do not increase susceptibility to septal defects in general because the VSD incidence was not different between the 2 intercrosses.

Second, atrioventricular septal defects (AVSDs) occur at a low but significant incidence in the Nkx2-5+/− C57Bl/6 × A/J F2 intercross (Figures 3 and 4). Nkx2-5 mutation causes the defect because none of 238 wild-type littermates from the same cross had an AVSD (P < 0.05). An A/J polymorphism enhances susceptibility because the defect is much rarer in the C57Bl/6 × FVB/N F2 intercross (P < 0.001). The A/J polymorphism appears dominant with low penetrance because 1 AVSD was found among 121 Nkx2-5+/− hearts from the backcross to C57BL/6.

Double-Outlet Right Ventricle Occurs Rarely in Nkx2-5+/− F2 Animals

Double-outlet right ventricle, in which both great arteries arise from the right ventricle and the aortic and mitral valves are separated by a muscular conus, has been reported in association with human Nkx2-5 mutation. Two Nkx2-5+/− F2 animals were found to have double-outlet right ventricle, 1 each from among 1552 C57Bl/6 × FVB/N and 1104 C57Bl/6 × A/J intercross hearts examined (Figure 4). The rarity of double-outlet right ventricle precludes conclusions about the role of modifier genes in its pathogenesis. We note, however, that defects associated with human Nkx2-5 mutation that are more common than double-outlet right ventricle in the general population like hypoplastic left heart syndrome have not been found in any Nkx2-5+/− mouse. Such defects may not have been found either because the sample size remains too small or because the inbred strains examined do not carry the relevant susceptibility alleles.

Genotypes at Multiple Modifier Loci Determine the VSD Phenotype

Membranous and muscular VSDs are found in Nkx2-5+/− hearts, but muscular VSDs are more common in the C57Bl/6 background (P < 0.0005; Figure 5). Which type of VSD develops appears to be determined by the cumulative effect of susceptibility genotypes at multiple modifier loci for either defect. We compared the incidence of each VSD type to the
fraction of the C57Bl/6 genome in the genetically heterogeneous F2 crosses, as expected from a mendelian distribution (Figure 5). There was a strong positive correlation for muscular VSD ($r = 0.91$, $P < 0.04$) and a negative correlation for membranous VSD ($r = -0.97$, $P < 0.006$). Thus, C57Bl/6, FVB/N, and A/J all carry recessive susceptibility alleles at multiple loci for either VSD phenotype, but in the mixed strain backgrounds, the additive effect of the C57Bl/6 susceptibility genotypes is less or greater than the other 2 strains for membranous and muscular VSD, respectively. Additional epistatic effects between loci may also influence the incidence of specific congenital heart defects, as shown by the segregation analyses below.

ASD and VSD Co-Occur More Frequently Than Expected by Chance in Nkx2-5+/− Hearts of Mixed Genetic Background

Polymorphic modifier genes clearly influence the development of specific types of defects in Nkx2-5 mutation. To test the hypothesis that the same polymorphism could increase susceptibility to 2 different defects, the incidence of hearts having both defects was compared with the product of the individual defect incidences in the population, ie, the chance or null hypothesis. VSD and ASD co-occur more often than expected by chance in the C57Bl/6 FVB/N intercross and the C57Bl/6 A/J intercross and backcross to C57Bl/6 (Figure 6A). In the 2 F2 intercrosses in which the sample sizes are large, ASD co-occurred with either muscular or membranous VSD more often than expected (Figure 6B). On the other hand, muscular and membranous VSD did not co-occur more often than expected. The co-occurrence of AVSD with other heart defects was not evaluated because of its low incidence. Therefore, some modifiers may increase the susceptibility to several types of heart defects in the presence of Nkx2-5 mutation, whereas others may affect the development of just 1.
Segregation Analysis of the Genetic Architecture of Common Septal Defects

To characterize the genetic architecture of modifiers that influence ASD and VSD susceptibility in Nkx2.5+/−/− animals, we examined 2 segregation models. The models focus on the C57Bl/6×FVB/N crosses because data from F2 backcrosses to both parental strains are available. How well the predictions of a model fit the observed data sets lower bounds on the number of modifiers and interactions that are involved in the pathogenesis of particular defects.

The first model, M1, postulates 2 loci, A and B, that do not interact. C57Bl/6 and FVB/N carry the recessive susceptibility alleles of A and B, respectively. The effects of susceptibility genotypes at A and B are estimated from the incidences in the C57Bl/6 strain and the F2 backcross to FVB/N. The expected incidences in the F2 intercross and C57Bl/6 backcross are then calculated from the model. Inclusion in the model of additional loci does not alter its predictions because the total effect of susceptibility alleles from 1 strain reduces to a single term. The predictions deviate significantly from the observed incidences of ASD and muscular VSD (P<0.0001) but approximate the membranous VSD incidences (P=0.14; Figure 7A through 7C). Therefore, at least 2 loci affect all 3 defect types.

Model M2 permits interaction between 2 modifier loci. In the simplest case, C57Bl/6 carries the recessive susceptibility alleles at 2 modifier loci, A and B, which interact synergistically. FVB/N carries the recessive susceptibility allele at a third locus, C. If the effect of A or B alone is negligible compared with their interaction, then the incidence of the defect in C57Bl/6 provides an estimate of the epistatic effect. On the basis of this model, the incidences attributed to the epistatic effect and locus C, as estimated from the F2 FVB/N backcross, match the observed incidences of muscular VSD in the F2 intercross and C57Bl/6 backcross (Figure 7A).

M2 underestimates the incidences of membranous VSD in the F2 intercross and C57Bl/6 backcross (P<0.0001; Figure 7B). M2 may not model the genetic architecture of membranous VSD either because epistatic interactions have no role (ie, M1 is correct) or because there are unaccounted interactions between C57Bl/6 and FVB/N alleles at other loci.

M2 poorly fits the ASD incidences in the F2 intercross and C57Bl/6 backcross (P<0.0001; Figure 7C). ASDs appear more genetically complex than VSDs. First, additional loci and interactions must be postulated to account for the >4-fold difference in the incidence of ASDs between the C57Bl/6 backcross and C57Bl/6. Inspection of the incidences in the crosses indicates that >2 modifier loci in each of the lines are likely be involved. Second, M1 and M2 predict the absence of defects in the F1 because homozygosity of a susceptibility allele at a modifier locus is assumed necessary for a defect. The assumption appears reasonable for muscular and membranous VSDs but not for ASDs. The presence of ASDs in the F1 suggests that heterozygosity at some loci confers susceptibility.

Taken together, the 2 segregation models indicate that ≥3 modifier loci and at least 1 epistatic interaction influence the susceptibility to muscular VSD and ASD in Nkx2.5+/−/− animals. At least 2 loci influence membranous VSD susceptibility. The loci and interactions inferred from the C57Bl/6×FVB/N crosses probably represent only a subset of all that exist among inbred mouse strains or the human population. A more precise estimate of the number of modifier loci in the mouse model, whether independent or epistatic, involved in any of the defects requires additional information such as genetic linkage analyses, which are underway.
Discussion

Normal cardiac development is critical for the survival of an individual. Any mechanism that could ensure normal development would increase the fitness of a species. In the short term, natural selection would eliminate major deleterious mutations. In contrast, stabilizing selection could promote the evolution of a versatile system that buffers the effects of genetic and environmental perturbations to the developing heart. The invariance of the normal heart form would then be enhanced.

Waddington described the general mechanism metaphorically as “canalization” in which development flows down channels to produce a highly invariant wild-type form. He considered the depth of the channels analogous to the degree of buffering, which he deduced to have a genetic basis. Crucial phenotypes are maintained about a stable optimum. Sufficiently great insults, however, decanalize an individual and reveal the influence of cryptic variants of modifier genes that do not affect the phenotype of genetically wild-type individuals. Investigation of modifier genes thus provides an alternative approach to the genetic pathways that shape a normal heart. Cryptic polymorphisms within them may give rise to much of the complexity of congenital heart disease.

The results of inbred strain crosses indicate that modifier genes contribute to the canalization and decanalization of cardiac development in the presence of Nkx2-5 mutation. A large fraction of Nkx2-5−/− animals in the C57Bl/6 background have ASDs and VSDs, whereas few among F1 hybrid crosses to FVB/N or A/J do. Defects recur in F2 crosses at a lower incidence than in C57Bl/6. We interpret this to mean that strain-specific polymorphisms of modifier genes alter the susceptibility of certain cardiac developmental pathways to Nkx2-5 mutation but do not affect the wild-type form. Complementation of susceptibility alleles in the F1 and homozygosity at some but not all modifier loci in the F2 contribute to the relative differences in defect incidence in the successive crosses. Furthermore, analysis of the incidences of ASD and VSD in the F2 crosses and C57Bl/6 suggests that modifier loci act independently of or epistatically with other loci. Thus, a protective allele at a modifier locus might directly reduce the risk of a defect or abrogate a synergistic interaction between loci that increases risk.

The surprisingly normal hearts of Nkx2-5−/− animals from the F1 hybrid crosses illustrate the important role of polymorphic modifier genes in ensuring the robustness of cardiac development. Polymorphisms likely exist against a variety of insults because reductions in heart defect incidence have also been observed in Tbx5 or Gata4 mutants in mixed compared with isogenic strain backgrounds. The greater heterogeneity in humans could provide even more genetic material to buffer against numerous potentially common insults like nutrient deprivation or congenital infection. For well-canalized traits, cryptic variation can accumulate in a population in the absence of a major perturbation because the phenotype is buffered against change. Natural selection resulting from diverse pressures in the wild could also maintain heterogeneity because normally cryptic polymorphisms may be protective or detrimental, depending on the context. The resulting genetic architecture could ensure that a fraction of the population survives almost any particular perturbation.

Recessive susceptibility alleles of modifier genes exist in each of the 3 inbred strains examined, given the presence of defects in the Nkx2-5−/− F2 progeny from all the parental backcrosses and intercrosses. Our statistical analysis of thousands of hearts reveals that modifier genes fixed for different variants in the inbred strains influence the development of specific types of defects in the presence of Nkx2-5 mutation. Relative to the other strain backgrounds, C57Bl/6 carries polymorphisms that increase the susceptibility to muscular VSD, and A/J carries polymorphisms that increase the susceptibility to ASD and AVSD. Modifier genes reside in pathways that affect the development of individual or multiple anatomic structures like the atrial and ventricular septum on the basis of the higher-than-expected coincidence of some defects. These observations inform how modifier loci and interactions could be mapped to define the genetic pathways leading from Nkx2-5 mutation to various types of defects.

Even genetic diseases thought to exhibit simple mendelian inheritance manifest with incomplete penetrance and varying phenotypes. The properties of genetic modifiers of the Nkx2-5 mutant phenotype help to explain the basis of the variable presentations of congenital heart disease. Ostensibly sporadic cases of congenital heart disease could result from germline or somatic mutations, teratogens, or purely stochastic events. Our results offer an alternative but not mutually exclusive possibility that polymorphisms of genetic modifiers can either suppress or promote the effect of a major mutation. Maximum genetic heterogeneity in the F1 is associated with near-complete suppression of deleterious phenotypes, leading to normal carriers. On the other hand, homozygosity at some loci in the F2 contributes to the manifestation of a specific defect among multiple potential phenotypes, which in a heterogeneous human population could appear like sporadic disease or pleiotropic defects.

This large study, which systematically varied the genetic background of a mouse model, offers insights into how genetic modifiers ensure normal cardiac development and influence the manifestation of congenital heart disease. Knowledge resulting from the characterization and identification of the modifiers in the mouse will almost certainly be relevant to human disease because pathways in cardiovascular development and physiology are strongly conserved. For example, dozens of quantitative trait loci that affect blood pressure are common between rodent models and human populations.

Cardiac development is robust. Appreciating this, one could conceivably mimic nature to reduce the heavy burden of congenital heart disease on children and their families.

Acknowledgments

We thank Goska Bielinska, James Cheverud, D.C. Rao, Alan Schwartz, David Wilson, and members of the Jay laboratory for their helpful input.

Sources of Funding

J.B. Winston was supported by a Ruth L. Kirschstein National Research Service Award from the Developmental Cardiology and Pulmonary Training Program (National Institutes of Health/National Heart, Lung, and Blood Institute T32 HL007873) and by the Lucille P. Markey Pathway in Human Pathobiology. Dr Jay is a Scholar of the Child Health Research Center of Excellence in Developmental
Biology at Washington University School of Medicine (National Institutes of Health K12-HD001487). This work was supported by the American Heart Association, Edward Mallinckrodt Jr. Foundation, Hartwell Foundation, and March of Dimes (Dr. Jay).

Disclosures
None.

References

**CLINICAL PERSPECTIVE**

Individuals who share the same underlying basis for congenital heart disease can have presentations ranging from normal to life-threatening. Understanding the basis of such wide variability could suggest prognostic and therapeutic strategies focused not on the causes but on the modifiers of disease. We thus characterized the effect of genetic modifiers on the incidence of heart defects associated with mutation of the cardiac transcription factor Nkx2-5. Quantitative analyses of multiple inbred mouse strain crosses reveal the profound effect of polymorphic genetic modifiers. Protective and susceptibility alleles of modifier loci direct the manifestation of 1 or more types of heart defects, suggesting that they affect the sensitivity of specific cardiac developmental pathways to a perturbation. The modifiers alter the risk of a particular phenotype either independently or via epistatic interactions with other loci. The results intertwine the genetic basis of health and congenital heart disease, providing a conceptual framework to understand common clinical observations related to incomplete penetrance and pleiotropy. We propose that stabilizing selection generated a diverse set of polymorphisms so that in a genetically heterogeneous population the predominant effect of modifier genes is to ensure the robustness of cardiac development.
Heterogeneity of Genetic Modifiers Ensures Normal Cardiac Development

_Circulation_. 2010;121:1313-1321; originally published online March 8, 2010; doi: 10.1161/CIRCULATIONAHA.109.887687

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/121/11/1313

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2010/03/04/CIRCULATIONAHA.109.887687.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/
Heterogeneity of genetic modifiers ensures normal cardiac development


SUPPLEMENTAL MATERIALS
Supplemental methods

Segregation analyses

Two segregation models are examined by comparison of their predicted and observed incidences of a defect in the F1 and F2 crosses. Statistically significant deviation from the model was determined in a $\chi^2$-test by $P < 0.05$.

The segregation analysis models assume that a defect may occur in animals with the $Nkx2-5^{+/+}$ genotype in combination with homozygosity at one or more modifier loci. Model M1 postulates two loci $A$ and $B$. C57Bl/6 and FVB/N carry the recessive, susceptibility alleles $A^{-}$ and $B^{-}$, respectively. The penetrance of a defect in an $Nkx2-5^{+/+}$ animal are $p_A$ for the $A^{+/+}B^{+/+}$ genotype, $p_B$ for $A^{+/+}B^{-/-}$, and $p_{AB}$ for $A^{-/-}B^{-/-}$. If no epistatic interaction or linkage exists between $A$ and $B$, then the penetrance $p_{AB}$ for the double homozygote $A^{-/-}B^{-/-}$ equals the sum of $p_A$ and $p_B$. Assuming no epistatic interaction in this model, $p_A$ and $p_B$ are estimated from the incidences in the C57Bl/6 strain and the F2 backcross to FVB/N. The expected incidences in the F2 intercross and backcross to C57Bl/6 are calculated from equations derived from the model (Supplemental Table). Inclusion in the model of additional loci does not alter its predictions because the total effect of susceptibility alleles from one strain reduces to a single term; for example, given $n$ loci at which C57Bl/6 carries the susceptibility alleles, $p_A = \sum p_{A,n}$.

Model M2 postulates multiple loci, two of which interact. Here C57Bl/6 carries the recessive susceptibility alleles at two modifier loci $A$ and $B$. FVB/N carries the recessive susceptibility allele at a third locus $C$. As in model M1, the penetrances of the single homozygotes are $p_A$, $p_B$, and $p_C$. The penetrance of the double homozygote $A^{-/-}B^{-/-}$ is
\( p_{AB} \). The other double and triple homozygote combinations are ignored for simplicity. If \( p_A \) and \( p_B \) are negligible compared to \( p_{AB} \), implying a synergistic interaction between \( A \) and \( B \), then \( p_{AB} \) would approximate the incidence of the defect in C57Bl/6. The incidences attributed to the \( A^{-}/B^{-} \) and \( C^{-} \) genotypes in the F2 backcross to C57Bl/6 and intercross are derived as shown (Supplemental Table).

**Supplemental Table**

<table>
<thead>
<tr>
<th>Cross</th>
<th>Model</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M1</td>
<td>M2</td>
</tr>
<tr>
<td>C57Bl/6</td>
<td>( p_A )</td>
<td>( F: p_A + p_B + p_{AB} )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( S: p_{AB} )</td>
</tr>
<tr>
<td>F1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Intercross</td>
<td>( F: 1/16 \times (3p_A + 3p_B + 4p_{AB}) )</td>
<td>( F: 1/16 \times (3p_A + 3p_B + p_{AB} + 4p_C) )</td>
</tr>
<tr>
<td></td>
<td>( S: 1/4 \times (p_A + p_B) )</td>
<td>( S: 1/16 \times (p_{AB} + 4p_C) )</td>
</tr>
<tr>
<td>C57Bl/6 backcross</td>
<td>( 1/2 \times p_A )</td>
<td>( F: 1/4 \times (p_A + p_B + p_{AB}) )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( S: 1/4 \times p_{AB} )</td>
</tr>
<tr>
<td>FVB/N backcross</td>
<td>( 1/2 \times p_B )</td>
<td>( 1/2 \times p_C )</td>
</tr>
</tbody>
</table>

The expected incidence of a defect in a particular cross is defined by a set of equations given by the segregation model. The penetrance terms \( p_A, p_B, p_C, \) and \( p_{AB} \) are estimated from the incidences in the C57Bl/6 strain or FVB/N backcross, depending upon the model and term. \( F \), full equation in which all terms described in the model are stated. \( S \), simplified equation in which assumptions regarding the interaction term \( p_{AB} \) are made, as explained in the Supplemental Methods.