Congenital Heart Disease

Interrogating Congenital Heart Defects With Noninvasive Fetal Echocardiography in a Mouse Forward Genetic Screen

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Background—Congenital heart disease (CHD) has a multifactorial pathogenesis, but a genetic contribution is indicated by heritability studies. To investigate the spectrum of CHD with a genetic pathogenesis, we conducted a forward genetic screen in inbred mice using fetal echocardiography to recover mutants with CHD. Mice are ideally suited for these studies given that they have the same four-chamber cardiac anatomy that is the substrate for CHD.

Methods and Results—Ethylnitrosourea mutagenized mice were ultrasound-interrogated by fetal echocardiography using a clinical ultrasound system, and fetuses suspected to have cardiac abnormalities were further interrogated with an ultrahigh-frequency ultrasound biomicroscopy. Scanning of 46 270 fetuses revealed 1722 with cardiac anomalies, with 27.9% dying prenatally. Most of the structural heart defects can be diagnosed using ultrasound biomicroscopy but not with the clinical ultrasound system. Confirmation with analysis by necropsy and histopathology showed excellent diagnostic capability of ultrasound biomicroscopy for most CHDs. Ventricular septal defect was the most common CHD observed, whereas outflow tract and atrioventricular septal defects were the most prevalent complex CHD. Cardiac/visceral organ situs defects were observed at surprisingly high incidence. The rarest CHD found was hypoplastic left heart syndrome, a phenotype never seen in mice previously.

Conclusions—We developed a high-throughput, 2-tier ultrasound phenotyping strategy for efficient recovery of even rare CHD phenotypes, including the first mouse models of hypoplastic left heart syndrome. Our findings support a genetic pathogenesis for a wide spectrum of CHDs and suggest that the disruption of left–right patterning may play an important role in CHD. (Circ Cardiovasc Imaging. 2014;7:31-42.)

Key Words: heart defects, congenital □ microscopy, acoustic

Chronic heart disease (CHD) is one of the most common birth defects, with ≈1% incidence in live births. The heritable nature of CHD is indicated by the increased recurrence risk for CHD and the observation that syndromic forms of CHD are often associated with chromosomal anomalies, such as 22q11 deletion in DiGeorge syndrome. However, investigations into the genetic basis of CHD have been challenging given that CHD is often sporadic. Even when there is strong evidence of heritability, there may be variable penetrance and variable expressivity. This could reflect the confounding effects of genetic heterogeneity in the human population. In addition, evidence suggests that environmental factors can contribute to CHD, indicating a multifactorial pathogenesis to CHD.

Clinical Perspective on p 42

Studies in mice have expanded our knowledge of genes that can cause CHD. Mice are well suited for modeling CHD because they have the same cardiac anatomy as humans, with 4-chamber hearts and distinct left–right asymmetries that provide separate pulmonary systemic circulation required for oxygenation of blood. These structures are the major targets of CHD. Although knockout mice have yielded new insights into the genetic pathogenesis of CHD, the analysis is limited to one gene at a time. In comparison, forward genetic screens using ethylnitrosourea (ENU) mutagenesis can greatly accelerate novel gene discovery. With ENU mutagenesis, every animal by design harbors many mutations. Thus, with focused phenotyping, gene discovery can proceed rapidly and without a priori gene bias. By conducting such screens in an inbred mouse strain background, the confounding effects of genetic heterogeneity could be minimized.1

We previously conducted a small-scale mouse forward genetic screen to recover CHD mutants using the Acuson clinical ultrasound system for noninvasive fetal echocardiography.2,3 Although echocardiography is the imaging modality of choice for diagnosing CHD, the poor imaging resolution of...
the Acuson restricted the ultrasound assessments to analysis of hemodynamic function. As a result, specific CHD diagnosis could only be made after necropsy and histopathology examination. Although CHD mutants were successfully recovered, these limitations compromised the efficacy of the screen, and some CHD phenotypes may be missed.

In this study, we incorporated the dual use of the Acuson and the Vevo2100 ultrahigh-frequency ultrasound biomicroscopy (UBM) for cardiovascular phenotyping to recover CHD mutants in a large-scale mouse mutagenesis screen. In contrast to our previous study, the Vevo2100 with its much higher resolution allowed direct diagnosis of structural heart defects. We diagnosed a wide spectrum of CHDs and recovered the first mouse model of hypoplastic left heart syndrome (HLHS), a rare phenotype never seen in mice previously.

Methods
ENU Mutagenesis and Mouse Breeding
All studies were conducted under an approved Institute Animal Care and Use Committee protocol of the University of Pittsburgh. C57BL/6J male mice were ENU-mutagenized as previously described with first generation(G1) male mice backcrossed to 4 to 6 G2 daughters, and the resulting G3 fetuses were ultrasound-scanned in utero. All offsprings from one G1 male mouse were tracked as a distinct pedigree.

Ultrasound Imaging and Doppler Echocardiography
Pregnant G2 dams were sedated with isoflurane and ultrasound-scanned using the Acuson Sequoia C512 with a 15-MHz transducer. Litters with abnormal fetuses were further scanned using the Vevo2100 UBM with a 40-MHz transducer for specific CHD diagnosis. The protocol used for ultrasound scanning, including the method for mapping the fetus position in utero, is described in the Methods section in the Data Supplement.

Necropsy, Microcomputed Tomography (Micro-CT)/Micromagnetic Resonance Imaging, and Histopathology Examinations
Fetuses or neonates were collected, fixed in 10% formalin, and then examined by necropsy, micro-CT or micromagnetic resonance imaging, and episcopic fluorescence image capture (EFIC), a histopathology examination. EFIC served as the gold standard for CHD diagnosis. All CHD diagnoses were reviewed by a panel of pediatric cardiologists and a pathologist (see the Data Supplement).

Efficacy of Ultrasound CHD Diagnosis
The efficacy of cardiovascular phenotyping with Acuson versus Vevo2100 was assessed by \( \chi^2 \) analysis, with significance set at \( P<0.05 \). The positive and negative predictive values of Vevo2100 ultrasound for CHD diagnosis were calculated as described in the Data Supplement. Statistical analysis was performed using SPSS 11.5 (SPSS Inc, Chicago, IL).

Results
Pregnant dams from ENU-mutagenized C57BL6/J mouse colony were ultrasound-scanned using the Acuson ultrasound system equipped with a 15-MHz transducer. The large 2-dimensional imaging window (25×20 mm) provided direct visualization of multiple fetuses, whereas the small footprint of the transducer facilitated rapid scanning. This allowed quick determination of the total number of embryos in a litter and their relative orientation in the uterine horn. Together with the spectral Doppler/color flow imaging capability, abnormal fetuses can be readily identified based on the finding of hemodynamic perturbation, hydrops, or growth restriction. The fewer litters with abnormal fetuses identified were further interrogated in a second-tier analysis using the higher-resolution Vevo2100 (30 um axial×75 um lateral resolution versus Acuson’s 300–500 um resolution). The Vevo2100 has a much smaller imaging window (15×14 mm) that allows visualization of only one fetus at a time, but its ultrahigh 2-dimensional resolution equipped with the same color flow imaging capability as the Acuson allows direct diagnosis of structural heart defects.

Vevo2100 scanning was conducted using a 40-MHz transducer with 3 diagnostic imaging planes, including sagittal (Figure 1A and 1A’), transverse four-chamber (Figure 1B and 1B’), and frontal (Figure 1C and 1C’) imaging planes. To determine heart and stomach situs, scans were conducted using 2 of 3 orthogonal imaging planes defined by the embryo’s body axes. Abnormal fetuses were rescanned on multiple days and, if deemed inviable to term, were harvested preterm. Subsequently, necropsy, micro-CT/micromagnetic resonance imaging, or histopathology examinations were
used to confirm CHD diagnosis. Typically, scans with the Acuson had ultrasound examination time of 55±16 (standard deviation) seconds per fetus for normal fetuses (median, 52 seconds; range, 34–100 seconds, based on 68 litters with 434 normal fetuses) and 64±15 (standard deviation) seconds per fetus for abnormal fetuses (median, 61 seconds; range, 32–105 seconds, based on 57 litters with 107 abnormal fetuses). The second-tier interrogation of abnormal fetuses using the Vevo2100 had a much longer examination time of 1049±297 (standard deviation) seconds per fetus (median, 1035 seconds; range, 600–1500 seconds, based on 27 abnormal fetuses in 20 litters). Overall, 20 litters were ultrasound-scanned per day, with ≤15 minutes required for a litter of 6 to 8 fetuses and longer scan times for litters with abnormal fetuses.

Fetuses were scanned from embryonic day (E) 13.5 to E18.5, with a mean of E15.2±1.4 (standard deviation) and median of E15.5. Because ventricular chamber and outflow tract (OFT) septation are not completed until E13.5 to 14.5, scanning at E13.5 to 15.5 should minimize false-positive CHD diagnosis that might reflect developmental delay. Also problematic with earlier scans is echogenicity of blood in younger embryos (due to nucleated erythrocytes), making it difficult to visualize the endocardial lumen, detracting from the otherwise high UBM 2-dimensional spatial resolution. Given these considerations, our fetal ultrasound screening was predominantly conducted at E14.5 to E15.5, with earlier scans conducted if dead embryos were observed at E14.5 to E15.5 (Figure 2A–2D).

Prevalence of Developmental Anomalies Identified by Ultrasound Phenotyping

Using the 2-tier ultrasound phenotyping strategy, we screened 46 270 G3 fetuses from 1381 G1 pedigrees (August 2010 to March 2012). We identified 2590 abnormal fetuses exhibiting a spectrum of cardiac and noncardiac defects (Table I and Figure I in the Data Supplement). Cardiac anomalies were found in 1722 fetuses (3.7%), which accounted for 66.5% of all developmental anomalies detected. In contrast, the incidences of extracardiac defects were much lower, ranging from 2% to 8% (Table I in the Data Supplement). These included craniofacial defects, limb anomalies, body wall closure defects, and laterality defects, including heterotaxy with discordant left–right heart and stomach positioning and situs inversus totalis (dextrocardia/dextrogastria; Table I in the Data Supplement).

Association of Prenatal Lethality and Growth Restriction With Cardiac Defects

The majority of fetuses with severe cardiovascular defects were observed to expire between E15.5 and term, whereas <20% of deaths at E14.5 were associated with CHD (Figure 2E). Cardiac anomalies were overall enriched in mutants with noncardiac defects (Table I in the Data Supplement). Thus, cardiac defects were found in 80.3% of mutants with body wall closure defects and 92.3% of mutants with heterotaxy. Growth retardation was also highly associated with cardiac defects, with 88.7% of growth-restricted fetuses exhibiting cardiac defects. More than 25% of fetuses (481 of...
1722; 27.9%) with cardiac defects died prenatally, showing the importance of prenatal screening. This included 15.1% (260 of 1722) fetuses found dead with follow-up ultrasound scans and 12.8% (221 of 1722) fetuses that were harvested prenatally given pending death was indicated with ultrasound presentations such as hydrops, pericardial effusion, dilated heart with bradycardia/arrhythmia, or severe inflow or outflow regurgitation (Figure 2A–2D; also see Movie IA–ID in the Data Supplement).

Recovery of a Wide Spectrum of Structural Heart Defects

A wide spectrum of CHDs was recovered from the ultrasound screen (Table 1). This included cardiac septation defects, OFT defects, left or right heart obstructive lesions,
coronary fistulas (Figure 3; Movie IIA–IID in the online-only Data Supplement), and cardiac situs anomalies. In 90 pedigrees, ≥2 G3 fetuses were observed to have the same structural heart defect phenotype. Such pedigrees with multiple affected fetuses were curated in the Mouse Genome Informatics database as part of the Bench to Bassinet collection of mutant mouse lines (http://www.informatics.jax.org/searchtool/Search.do?query=b2b&submit=Quick+Search), and sperm from G1 male mice was cryopreserved at the Jackson Laboratory (Table II in the Data Supplement). The most common structural heart defect observed was ventricular septal defect (VSD; 50%), whereas among complex CHD phenotypes, OFT defects were the most common (36.1%; Table 1).

**OFT Anomalies**

The most common OFT anomaly was double outlet right ventricle (DORV)/overriding aorta (21.3%). Shown in Figure 4 is a fetus exhibiting DORV with anterior placement of the aorta and subpulmonary VSD, a phenotype clinically referred to as Taussig–Bing subtype of DORV. This mutant also exhibited heterotaxy with abnormal rightsided rather than leftsided stomach (Movie IIIA–IIIE in the Data Supplement). Also observed was persistent truncus arteriosus (Figure 5A–5E) and transposition of the great arteries (Table 1).

Given the very small size of the fetal mouse heart, even with the higher resolution of the Vevo2100, DORV was not reliably distinguished from overriding aorta, nor was persistent truncus arteriosus always distinguishable from pulmonary atresia. Therefore, we grouped DORV with overriding aorta and persistent truncus arteriosus with pulmonary atresia (Table 1).

**Atrioventricular Septal Defects and Right–Left Heart Obstructive Lesions**

Atrioventricular septal defects were also commonly observed in our screen, especially in conjunction with OFT anomalies (Figure 6; Movie IVA–IVE in the Data Supplement; Table 1). Secundum atrial septal defect and foramen ovale were not distinguishable and were not tracked. Among right heart obstructive lesions, pulmonary stenosis was the most common. We observed some cases of hypoplastic right heart syndrome and also hypoplastic tricuspid (Figure 7A–7F). Among left heart obstructive lesions, the most common was aortic stenosis/coarctation (Figure 7G–7K). Most unexpected was the finding of fetuses with HLHS (Figure 8).

**Hypoplastic Left Heart Syndrome**

HLHS is one of the rarest phenotypes recovered in our screen; 4 HLHS fetuses were identified from >46,000 fetuses scanned. This was derived from 3 independent G1 pedigrees. HLHS is characterized by underdevelopment of the left side of the heart, including hypoplasia of the left ventricle, mitral valve, aorta, and aortic arch (Figure 8). This phenotype has never been observed in mice previously. In mutant line 635, ultrasound diagnosed 1 fetus with HLHS at E14.5, which was confirmed with follow-up ultrasound analysis on subsequent days. Color flow imaging showed reverse aortic flow from the descending to ascending aorta (Figure 8A–8C). Mitral valve atresia was indicated by lack of color inflow, whereas...
2-dimensional imaging showed a very small left ventricle with little or no lumen (Figure 8D). This mutant was stillborn, and necropsy revealed hypoplastic ascending aorta (Figure 8E), with subsequent EFIC histopathology showing small left ventricle with almost no lumen, hypoplastic ascending aorta, and hypoplastic mitral valve. These findings together confirmed the ultrasound diagnosis of HLHS (Figure 8F and 8G; Movie IV A–IVC in the Data Supplement).

Heritability of the HLHS phenotype was demonstrated in this mutant line with the recovery of 2 HLHS mutants from 62 offsprings screened. In the other 2 mutant lines, only a single HLHS fetus was found among 18 and 29 offsprings interrogated, respectively. Given the rarity of HLHS, all 3 HLHS mutant lines were curated in Mouse Genome Informatics and sperm was cryopreserved at the Jackson Laboratory.

**Congenital Heart Defects Associated With Laterality Defects**

Because visceral organ situs can be determined by ultrasound phenotyping with the Vevo2100, we assessed situs anomalies as part of the routine CHD phenotyping workflow. Surprisingly, more than half (49 of 90) of the mutant lines with CHD exhibited laterality defects (Table II in the online-only Data Supplement). This included fetuses with situs inversus totalis with mirror symmetrical visceral organ situs, and heterotaxy with randomized visceral organ situs. Nearly all heterotaxy mutants had complex CHD, which was readily detected by echocardiography (see example in Figure 4). In contrast, mutants exhibiting situs inversus totalis generally did not have CHD, although some had VSDs.

**Efficacy of Congenital Heart Defect Diagnosis With UBM Versus Acuson**

We compared the efficacy of the Acuson versus the UBM in CHD diagnosis by examining the ultrasound findings obtained in 1457 abnormal fetuses using both ultrasound systems (Table 1). UBM was significantly better in detecting structural heart anomalies, such as septal defects ($P<0.01$), OFT defects ($P<0.01$), left heart obstructive lesions ($P<0.01$), right heart obstructive lesions ($P<0.01$), and cardiac situs anomaly ($P<0.01$). We noted that some structural heart defects were only detected with the UBM, including HLHS,
hypoplastic right heart syndrome, mitral valve atresia/stenosis, tricuspid atresia/stenosis, mesocardia/dextrocardia, and transposition of the great arteries (Table 1). However, there was no difference in the efficiency for detection of hemodynamic perturbations, such as regurgitant flow or velocity increase in the inflow tract or OFT, and other nonspecific cardiac indications encompassing arrhythmia, hydrops, or pericardial effusion (Table 1).

Accuracy of Congenital Heart Defect Diagnoses by Vevo2100 Ultrasound

The efficacy of Vevo2100 ultrasound phenotyping of CHD was evaluated using EFIC histopathology as the gold standard for structural heart defect diagnosis (Table 2). In total, 524 fetuses interrogated by 2-tier ultrasound screening were evaluated by EFIC imaging (Figure II in the Data Supplement). This included 277 fetuses identified with cardiac lesions, 161 fetuses identified as without CHD from among these same litters with affected fetuses, and 86 fetuses initially identified with abnormal findings by Acuson but subsequently diagnosed as without CHD by Vevo2100. This combined analysis of 277 fetuses with cardiac lesions and 247 fetuses without cardiac defects showed negative predictive value (NPV) ranging from 80.9% to 100% (Table 2; Table III in the Data Supplement). In contrast, positive predictive value (PPV) varied from 60% to 100%, with the lowest associated with aortic anomalies, tricuspid anomalies, and coronary fistulas (Table 2; Table III in the Data Supplement), indicating that the latter defects were missed more frequently.

Overall, our analysis showed excellent diagnostic capability of Vevo2100 ultrasound for most CHDs. For septal defects, PPV (95.7%) was high, but NPV (80.9%) was lower. This mainly reflects the failure to detect very small VSDs. For OFT defects, although NPV (93.8%) was high, we observed PPV of only 85.4%. Thus, among 130 OFT defects diagnosed by EFIC, 25 were missed by Vevo2100 ultrasound scans; these were largely comprised of transposition of the great arteries and DORVs. We found HLHS as the only CHD diagnosis with 100% accuracy (Table 2; Table III in the Data Supplement). For cardiac situs, PPV of 100% was observed with 99% NPV, a reflection of the high accuracy of Vevo2100 ultrasound in determining cardiac situs. In 5 of 23 cases of dextrocardia missed by Vevo2100 ultrasound, all were associated with situs inversus totalis.

Discussion

Our study showed the efficacy of noninvasive mouse fetal ultrasound imaging with the Acuson and Vevo2100 ultrasound systems for cardiovascular phenotyping. The Acuson allowed high-throughput screening for quick identification of abnormal fetuses, with subsequent UBM interrogation for specific CHD diagnosis. Scan time of ≈18 minutes per abnormal fetus was required for the UBM but only 1 minute per fetus with the Acuson. Previous studies by Ji and Phoon6 using another UBM instrument reported scan time of ≈1 hour per litter, consistent with our observation. The ultrasound system used in previous studies could not diagnose specific structural heart defects, given the lack of color flow Doppler function, but nevertheless, their hemodynamic assessments showed that the outflow cushions performed a valve-like function critical for the survival of early mouse embryo.7

Using Vevo2100 equipped with color flow Doppler, although we observed a NPV >93% for most CHDs, PPV varied with low diagnostic capability for valvular defects, coronary artery fistulas, and small VSDs. These findings are similar to those reported in human fetal ultrasound studies.8 The comparison of CHD diagnosis achieved with Vevo2100 versus postmortem micro-CT imaging showed similar high diagnostic accuracy for most CHDs, except for aortic arch anomalies and situs defects.9 Current UBM technology...
cannot visualize the small aortic arch vessels, but these can be detected by contrast-enhanced micro-CT. Micro-CT also gave high accuracy in the detection of situs anomalies because the animal’s left–right axis can be predictably fixed during CT scanning. In contrast, fetus orientation in utero during ultrasound scanning may be highly variable.

Recovery of CHD Mutants by Fetal Ultrasound Phenotyping

Our screen encompassing >46,000 fetuses is one of the largest CHD screens to date. We observed a 3.7% incidence of cardiac defects and curated and cryopreserved >90 mutant mouse lines with CHD. We observed that CHD is highly associated with growth retardation and extracardiac anomalies. This is consistent with clinical studies showing intrauterine growth restriction as a significant risk factor for CHD and the report that CHD is linked with chromosomal abnormalities, suboptimal growth, and extracardiac malformations. We found that >25% of fetuses with cardiac defects died prenatally, with many expiring between E15.5 and E16.5, equivalent to 8- to 12-week gestation in human embryos. Interestingly, clinical studies have shown higher fetal death associated with CHD in human fetuses <15 weeks and an overall increase in CHD incidence in fetuses dying before term.

With fetal ultrasound imaging, we recovered at-risk fetuses that would otherwise be missed in a postnatal screen. Moreover, with the identification of fetuses with CHD by ultrasound, we could exercise more caution in recovering stillborn pups, which might otherwise be cannibalized by the mother. We note another study, in which CHD mutants were screened with postnatal collection of stillborn pups, followed by phenotyping using histopathology in a mouse mutagenesis screen. Although CHD mutants can be recovered in this manner, such screen is not high throughput, and a significant fraction of CHD mutants would be missed. Our analysis of stillborn pups showed that only 13% had CHD, most of which were isolated VSDs (unpublished observations).

Wide Spectrum of CHD Recovered From the Mutagenesis Screen

The most prevalent cardiac defect found was VSD, observed in 50% of CHD mutants, half of which were isolated VSDs. VSD is also the most common CHD observed clinically,
suggesting that a large number of genes have a role in VSD.\textsuperscript{15} OFT anomalies (36%) and atrioventricular septal defects (19%) were the most common complex CHDs. Among OFT defects, DORV was the most common, accounting for >50% of OFT anomalies observed. DORV is also one of the most common OFT anomalies seen in patients with CHD. Surprisingly, we did not observe Tetralogy of Fallot, but it has been observed in knockout mouse models, albeit with variable penetrance.\textsuperscript{16} Another unexpected finding was the large number of CHD mutants with laterality defects, with dextrocardia/mesocardia seen in 11% of CHD mutants. In comparison, human clinical studies\textsuperscript{17} showed that laterality defects account for only 3% of all CHDs. As our study showed, heterotaxy mutants often died prenatally from complex CHDs; human conceptuses with complex CHD associated with heterotaxy may be underrepresented in the clinical population. Studies have shown an excess of complex lesions in human fetuses with CHD that did not survive to term.\textsuperscript{15} Together, these findings suggest that genes regulating left–right patterning may play an important role in complex CHD.

**Mouse Models of HLHS**

HLHS was the rarest CHD phenotype recovered, with an incidence of 1.1% among CHD mutants in our screen. HLHS is also clinically rare, the prevalence varying depending on the patient population.\textsuperscript{8,15} The recovery of HLHS mutants was made possible by both the scale of our screen and the efficacy of the UBM in yielding hemodynamic and structural information. Although HAND-1–null mutant mice have a hypoplastic left ventricle,\textsuperscript{18} they do not exhibit all 3 essential features of HLHS. It should be noted that our screen was designed to recover recessive mutations, but only 4 HLHS fetuses were recovered from >100 fetuses ultrasound-scanned in 3 independent pedigrees, which is inconsistent with a monogenic recessive pathogenesis. Clinical studies have shown that
HLHS is genetically heterogeneous and possibly with multigenic pathogenesis.19

Genetic Contribution to CHD

Findings from our forward genetic screen support a genetic pathogenesis for a wide spectrum of CHDs, including VSD, the most common cardiac defect, and HLHS, one of the most severe CHDs. Although our screen was conducted using a 2-generation backcross breeding scheme designed to recover recessive mutations, every G1 pedigree is estimated to have >20 deleterious mutations.20 This provides a genomic context for modeling some of the complex genetics of CHD seen in the human population, including CHD with a multigenic pathogenesis. Perhaps this accounts for our success in recovering HLHS mutants.

One CHD phenotype not observed in our screen was Ebstein’s anomaly, a CHD involving malformation of the tricuspid valve. It is one of the rarest CHDs, with a prevalence of 1 in 200,000. Because this CHD phenotype should be readily detectable by UBM, the failure to find Ebstein’s anomaly could simply reflect insufficient sample size. Alternatively, strain background effects may be a contributing factor. Various clinical and animal model studies have indicated a genetic pathogenesis for Ebstein’s anomaly,21 but this CHD also has been linked to environmental exposures,22 suggesting the possible involvement of genetic and environmental factors.

Limitations

Although fetal ultrasound scans can be initiated in early gestation (<E15.5), care must be exercised to ensure that anomalies detected do not represent developmental delay. Yet another limitation is the potential failure to recover isolated small VSDs and vascular anomalies such as coronary fistula and valvular defects. Although such defects could be readily diagnosed by the UBM, they may be missed by the initial Acuson scans in a 2-tier screen.

Conclusions

We showed that noninvasive fetal echocardiography can serve as a sensitive and high-throughput imaging modality for CHD diagnosis. Our forward genetic screen using fetal ultrasound imaging has provided evidence of genetic pathogenesis for a wide spectrum of CHDs, including HLHS. Although our breeding scheme was optimized for detection of recessive mutations, our findings suggest a more complex multigenic
pathogenesis for HLHS and, perhaps, other CHDs. We propose that ENU mutagenesis using inbred mice can provide an ideal genomic context to interrogate and model the complex genetics of human CHDs.

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Disclosures

None.

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typing of fetal mice by noninvasive high-frequency ultrasound facilitates recovery of ENU-induced mutations causing congenital cardiac and extra-
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10. Perez-Delboy A, Simpson LL. Prenatal sonographic diagnosis of congeni-
Congenital heart disease (CHD) is one of the most common birth defects. Investigations into the genetic pathogenesis of CHD have been challenging given the sporadic nature and variable penetrance and expressivity of CHD. Further complications come from indications that environmental factors also contribute to CHD. To interrogate the genetic pathogenesis of CHD, we undertook a forward genetic screen in chemically mutagenized C57BL6 inbred mice to recover CHD mutants. A 2-tier ultrasound screening protocol was developed involving initial scans using the Acuson clinical ultrasound system to identify fetuses with cardiac defects, followed by detailed scans of abnormal fetuses for CHD diagnosis using the ultrahigh-frequency Vevo2100 ultrasound biomicroscopy. We scanned 46,270 fetuses and identified 1722 with cardiac defects, >25% of which died prenatally. A wide spectrum of CHDs was observed, most of which could be diagnosed using Vevo2100 but not with Acuson. The confirmation of CHD diagnosis by necropsy and histopathology showed excellent diagnostic capability of ultrasound biomicroscopy for most CHDs. Ventricular septal defect was the most common CHD observed, whereas outflow tract and atrioventricular septal defects were the most prevalent complex CHD. Laterality defects including heterotaxy were observed at surprisingly high incidence. The rarest CHD found was hypoplastic left heart syndrome, a phenotype never seen in mice previously. Our findings support a genetic pathogenesis for a wide spectrum of CHDs and suggest that mouse fetal echocardiography together with forward genetic screens can be used to interrogate and model the complex genetics of human CHD.
Interrogating Congenital Heart Defects With Noninvasive Fetal Echocardiography in a Mouse Forward Genetic Screen

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SUPPLEMENTAL MATERIAL

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SUPPLEMENTAL METHODS

Ultrasound Imaging and Doppler Echocardiography

When fetal ultrasound was performed, the mother’s bladder was used as an anatomic landmark to locate fetuses proximally from the cervix to more distally in the uterine horn on the left as L1,2,3, etc., and on the right as R1,2,3, etc. The imaging modalities used included B-mode imaging, and color flow and spectral Doppler, and M-mode imaging as previously described\textsuperscript{1,2}. The initial Acuson scan was used to determinate litter size, orientation of fetuses and developmental staging of the fetuses with crown to rump length and fetus area measurements. Crown-to-rump length of fetus > two standard deviations below the mean compared to age-matched normal fetuses were considered growth restricted\textsuperscript{3}.

Analysis of Positive and Negative Predictive Value with Vevo2100 Ultrasound Imaging

Fetuses examined by the Acuson-Vevo2100 two-tier ultrasound screen were subsequently analyzed by EFIC histology as the gold standard for CHD diagnosis. This included fetuses that were Vevo ultrasound diagnosed with CHD, fetuses Vevo ultrasound diagnosed as without CHD from the same litters with fetuses Vevo diagnosed with CHD, and fetuses from entire litters that were Vevo ultrasound diagnosed as without CHD. Provisional CHD diagnoses were made based on the ultrasound findings. Then after the EFIC imaging data was obtained, a panel of pediatric cardiologists and a pediatric pathologist reviewed the data and made consensus CHD diagnoses. The CHD diagnosis determined by EFIC imaging was compared to the diagnosis made by Vevo ultrasound imaging to determine the number of true positives, false positives, true negatives and false negatives. Using these numbers, the positive predictive value (PPV) and negative predictive value (NPV) were calculated. The
PPV = (number of true positives) / (number of true positives + number of false positives); the
NPV = (number of true negatives) / (number of true negatives + number of false negatives).
Table S1. Developmental anomalies detected by fetal ultrasound

<table>
<thead>
<tr>
<th>Defects</th>
<th>Pedigrees*</th>
<th>G2 Females†</th>
<th>Total Fetuses‡</th>
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<th>Cardiac Defects‖</th>
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<td>20.9%</td>
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<tr>
<td>Prenatal Lethality</td>
<td>523 (37.9%)</td>
<td>871 (15.2%)</td>
<td>1,189 (2.6%)</td>
<td>45.9%</td>
<td>481 (40.5%)</td>
<td>20.0%</td>
</tr>
<tr>
<td>Growth Retarded</td>
<td>130 (9.4%)</td>
<td>168 (2.9%)</td>
<td>204 (0.4%)</td>
<td></td>
<td>181 (88.7%)</td>
<td>17.7%</td>
</tr>
<tr>
<td>Hydrops</td>
<td>303 (21.9%)</td>
<td>425 (7.4%)</td>
<td>635 (1.4%)</td>
<td>24.5%</td>
<td>271 (42.7%)</td>
<td>20.8%</td>
</tr>
<tr>
<td>Craniofacial/Limb Defects</td>
<td>109 (7.9%)</td>
<td>143 (2.5%)</td>
<td>221 (0.5%)</td>
<td>8.5%</td>
<td>143 (64.7%)</td>
<td>20.6%</td>
</tr>
<tr>
<td>Body Wall Defects</td>
<td>34 (2.5%)</td>
<td>41 (0.7%)</td>
<td>61 (0.1%)</td>
<td>2.4%</td>
<td>49 (80.3%)</td>
<td>22.1%</td>
</tr>
<tr>
<td>Heterotaxy/ Situs Defects</td>
<td>30 (2.2%)</td>
<td>41 (0.7%)</td>
<td>52 (0.1%)</td>
<td>2.0%</td>
<td>48 (92.3%)</td>
<td>19.5%</td>
</tr>
</tbody>
</table>

*Number G1 pedigrees screened. Number in parentheses represents percent pedigrees with indicated defects in total pedigrees screened.
†Number G2 females scanned with fetuses exhibiting the indicated defects. Number in parentheses represents percent G2 females with indicated defects in total G2 females screened.
‡Number in parentheses represents percent of fetuses with indicated defects in total fetuses screened.
§Percent of abnormal fetuses with the indicated defects.
‖Fetuses with indicated developmental anomaly in conjunction with cardiac defects. Percent = fetuses with indicated anomaly with cardiac defects/total fetuses with indicated anomaly.
#Percent of fetuses with indicated defects amongst all fetuses in the affected litters.
Table S2. Congenital Heart Disease Mutant Lines Archived*

<table>
<thead>
<tr>
<th>Congenital Heart Defect Phenotype</th>
<th>No. Lines†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lines with CHD</td>
<td>90</td>
</tr>
<tr>
<td>Laterality Defects</td>
<td>49</td>
</tr>
<tr>
<td><strong>Septal Defects</strong></td>
<td></td>
</tr>
<tr>
<td>Ventricular Septal Defect</td>
<td>64</td>
</tr>
<tr>
<td>Atrial Septal Defect</td>
<td>30</td>
</tr>
<tr>
<td>Atrioventricular Septal Defect</td>
<td>35</td>
</tr>
<tr>
<td><strong>Outflow Tract Malalignments</strong></td>
<td></td>
</tr>
<tr>
<td>Double Outlet Right Ventricle</td>
<td>41</td>
</tr>
<tr>
<td>Persistent Truncus Arteriosus/Pulmonary atresia</td>
<td>16</td>
</tr>
<tr>
<td>Transposition of Great Arteries</td>
<td>10</td>
</tr>
<tr>
<td><strong>Aortic Stenosis/Coarctation</strong></td>
<td>10</td>
</tr>
<tr>
<td>Pulmonary Stenosis</td>
<td>16</td>
</tr>
<tr>
<td>Tricuspid Atresia</td>
<td>6</td>
</tr>
<tr>
<td>Hypoplastic Left Heart Syndrome</td>
<td>3</td>
</tr>
<tr>
<td>Hypoplastic Right Heart Syndrome</td>
<td>4</td>
</tr>
<tr>
<td><strong>Arch Anomaly</strong></td>
<td></td>
</tr>
<tr>
<td>Right aortic arch</td>
<td>22</td>
</tr>
<tr>
<td>Interrupted Aortic Arch</td>
<td>8</td>
</tr>
<tr>
<td>Vascular Ring</td>
<td>3</td>
</tr>
<tr>
<td><strong>Coronary Fistula</strong></td>
<td>5</td>
</tr>
</tbody>
</table>

*All mutant lines curated in the Mouse Genome Informatics Database (www.informatics.jax.org) and with sperm cryopreserved at the Jackson Laboratory.†Mutant lines may be represented in multiple CHD categories.
Table S3. Accuracy of Vevo2100 Ultrasound Diagnosis of Congenital Heart Disease*

<table>
<thead>
<tr>
<th>CHD diagnosis</th>
<th>Values Obtained Without the Additional 86 Normal Fetuses</th>
<th>Values Obtained With the Additional 86 Normal Fetuses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PPV</td>
<td>NPV</td>
</tr>
<tr>
<td>Septal Defects†</td>
<td>95.7%</td>
<td>75.6%</td>
</tr>
<tr>
<td>Outflow Tract Defects‡</td>
<td>85.4%</td>
<td>92.1%</td>
</tr>
<tr>
<td>HLHS</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>HRHS</td>
<td>77.8%</td>
<td>99.5%</td>
</tr>
<tr>
<td>MS/MA</td>
<td>75%</td>
<td>99.5%</td>
</tr>
<tr>
<td>AS/AA/COA</td>
<td>60%</td>
<td>95.3%</td>
</tr>
<tr>
<td>Tricuspid Hypoplasia/Atresia</td>
<td>60%</td>
<td>99.1%</td>
</tr>
<tr>
<td>Pulmonary Stenosis</td>
<td>90%</td>
<td>96.5%</td>
</tr>
<tr>
<td>Coronary Artery Fistula</td>
<td>71.4%</td>
<td>97.9%</td>
</tr>
<tr>
<td>Cardiac Situs Defects</td>
<td>100%</td>
<td>99%</td>
</tr>
</tbody>
</table>

* Grey columns: Total number fetuses/pups analyzed = 438. It included 277 identified with CHD + 161 littermates of affected fetuses but ultrasound identified as without CHD.
Clear columns: Total number fetuses/pups analyzed = 524. It included 277 identified with CHD + 161 littermates of affected fetuses but ultrasound identified as without CHD + 86 fetuses from litters that were ultrasound identified as without CHD.
SUPPLEMENTAL FIGURES

Figure S1 Summary of Ultrasound Screen

Acuson scanned 46,270 fetuses

2,590 abnormal fetuses

- 868 fetuses with non-cardiac defects
- 1,722 fetuses with cardiac defects

1,457 fetuses with cardiac defects
Acuson and Vevo scanned

- 352 fetuses identified with CHD

277 fetuses with CHD recovered and EFIC analyzed

1,105 fetuses identified with non-specific cardiac presentations

265 fetuses with cardiac defects
Acuson scanned only

75 fetuses resorbed or cannibalized
Figure S2. EFIC Imaging Analysis to Assess Accuracy of Vevo2100 Diagnosis of CHD

Note fetuses analyzed by EFIC imaging for CHD confirmation are denoted in the blue boxes.
SUPPLEMENTAL REFERENCES


VIDEO LEGENDS

VIDEO 1
Video 1A: A fetus was found with hydrops at E13.5 by the Acuson
Video 1B: Hydrops (Arrow) and pericardial effusion (labeled PE with arrow) were detected by further UBM scanning. (PE=pericardial effusion)
Video 1C: The UBM color flow mapping in transverse view showed bidirectional shunt and regurgitation of AVSD.
Video 1D: Acuson scan of the same fetus one day later (E14.5) showed the affected fetus had died, as it exhibited no heart beating.

VIDEO 2
Video 2A: The Acuson imaging in sagittal view detected abnormal blood flow (blue color flow) during diastole.
Video 2B: The UBM 2D imaging in frontal view revealed a gap or hole (labeled CF with arrow) in the ventricular septum. (CF=coronary artery fistula).
Video 2C: UBM color flow mapping in frontal view detected an abnormal vessel (blue flow stream) during diastole (arrow), which originated from the root of the aorta and exited into the RV, suggesting it is a coronary artery fistula.
Video 2D: Micro-MRI confirmed UBM diagnosis of coronary artery fistula.

VIDEO 3
Video 3A: Levocardia as seen in the transverses view by 2D imaging with the Vevo2100.
Video 3B: Right-sided stomach seen in the transverse view by 2D imaging with the Vevo2100.
Video 3C: Vevo2100 color flow mapping in the sagittal view revealed anterior positioning of the aorta, posterior pulmonary artery, and outflow regurgitation, which together indicated transposition of the great arteries.
Video 3D: Vevo2100 color flow mapping in the sagittal view showed two overlapping red flow streams representing anterior aorta with aortic regurgitation, posterior pulmonary artery emerging from the RV, and a subpulmonary VSD. Together these presentations would suggest DORV of the Taussig-Bing type.
Video 3E: EFIC 2D serial image stack of the same fetus confirmed DORV of the Taussig-Bing type.
VIDEO 4

**Video 4A:** 2D imaging in the transverse view with the Vevo2100 showed atrioventricular septal defect. (CV= common atrioventricular valve)

**Video 4B:** Color flow mapping using the Vevo2100 showed atrioventricular septal defect with regurgitation in the transverse view.

**Video 4C:** Pulmonary artery and majority of aorta emerge from the RV with hypoplastic PA seen by UBM 2D imaging in sagittal view.

**Video 4D:** The UBM color flow mapping in the sagittal view was diagnosed as pulmonary artery and majority of aorta connected with the RV, but no obvious flow from the PA and a possible subaortic VSD, which together indicated DORV with severe pulmonary stenosis.

**Video 4E:** EFIC movie displayed DORV with severe pulmonary stenosis and AVSD. There was also a hypoplastic branch pulmonary artery with PDA.

VIDEO 5

**Video 5A:** Vevo2100 color flow imaging in the sagittal view revealed hypoplastic aorta with reversal of aortic blood flow, indicating severe aortic stenosis. Note blood flow from the descending aorta into the ascending aorta, with only very small flow through the aortic valve.

**Video 5B:** Hypoplastic LV as seen by Vevo2100 2D imaging in the transverse view.

**Video 5C:** Quicktime movie of EFIC serial 2D image stack showed hypoplastic aorta with severe aortic stenosis, and hypoplastic left ventricle with hypoplastic mitral valve, which together comprises HLHS.