Cardiovascular phenotyping of fetal mice by noninvasive high-frequency ultrasound facilitates recovery of ENU-induced mutations causing congenital cardiac and extracardiac defects

Yuan Shen,1 L. Leatherbury,1,2 J. Rosenthal,1 Qing Yu,1 M. A. Pappas,1 A. Wessels,3 J. Lucas,3 B. Siegfried,1 B. Chatterjee,1 Karen Svenson,4 and C. W. Lo1

1Laboratory of Developmental Biology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland; 2Pediatric Cardiology, Children’s National Medical Center, Washington, District of Columbia; 3Department of Anatomy and Cell Biology, Medical University of South Carolina, Charleston, South Carolina; and 4The Jackson Laboratory, Bar Harbor, Maine

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Shen, Yuan, L. Leatherbury, J. Rosenthal, Qing Yu, M. A. Pappas, A. Wessels, J. Lucas, B. Siegfried, B. Chatterjee, Karen Svenson, and C. W. Lo. Cardiovascular phenotyping of fetal mice by noninvasive high-frequency ultrasound facilitates recovery of ENU-induced mutations causing congenital cardiac and extracardiac defects. Physiol Genomics 24: 23–36, 2005. First published September 20, 2005; doi:10.1152/physiolgenomics.00129.2005.—As part of a large-scale noninvasive fetal ultrasound screen to recover ethylnitrosourea (ENU)-induced mutations causing congenital heart defects in mice, we established a high-throughput ultrasound scanning strategy for interrogating fetal mice in utero utilizing three orthogonal imaging planes defined by the fetus’ vertebral column and body axes, structures readily seen by ultrasound. This contrasts with the difficulty of acquiring clinical ultrasound imaging planes which are defined by the fetal heart. By use of the three orthogonal imaging planes for two-dimensional (2D) imaging together with color flow, spectral Doppler, and M-mode imaging, all of the major elements of the heart can be evaluated. In this manner, 10,091 ENU-mutagenized mouse fetuses were ultrasound scanned between embryonic days 12.5 and 19.5, with 324 fetuses found to die prenatally and 425 exhibiting cardiovascular defects. Further analysis by necropsy and histology showed heart defects that included conotruncal anomalies, obstructive lesions, and shunt lesions as well as other complex heart diseases. Ultrasound imaging also identified craniofacial/head defects and body wall closure defects, which necropsy revealed as encephalocele, holoprosencephaly, omphalocele, or gastrochisis. Genome scanning mapped one ENU-induced mutation associated with persistence truncus arteriosus and holoprosencephaly to mouse chromosome 2, while another mutation associated with cardiac defects and omphalocele was mapped to mouse chromosome 17. These studies show the efficacy of this novel ultrasound scanning strategy for noninvasive ultrasound phenotyping to facilitate the recovery of ENU-induced mutations causing congenital heart defects and other extracardiac anomalies.

echocardiography; congenital heart defects; mouse mutagenesis; ethylnitrosourea

OVER THE PAST DECADE, forward and reverse genetic approaches have been successfully applied to generating mouse models for studying the genetic basis for congenital heart defects (4, 23, 31). Chemical mutagenesis can be used as a nonbiased approach for recovering novel genes as well as novel alleles of known genes that may contribute to congenital heart defects (5, 30, 35). For recovering mutations causing congenital heart disease, the screening method should provide the means to evaluate both cardiovascular structure and function, and ideally this should be carried out in a noninvasive manner. Given that mutagenesis by necessity is random, the overall success of such screens is largely dependent on having a screening method that is both high throughput and sensitive. A variety of noninvasive imaging techniques are now available for cardiovascular phenotyping, including conventional ultrasound (12, 16, 22, 34, 46), ultra-high-frequency ultrasound (10, 33, 46, 49), and magnetic resonance imaging (MRI) (27). These imaging methods can provide quantitative assessments of both cardiovascular structure and function. Among these three methods, only clinical ultrasound has been used for noninvasive cardiovascular phenotyping of fetal mice (12, 16, 55).

The ability to phenotype mouse fetuses by in utero ultrasound is highly advantageous, since horizontal studies can be carried out to examine the onset and progression of cardiovascular disease in the developing fetus. In addition, because mice with serious congenital heart defects often die before term, prenatal fetal ultrasound represents the only method for identifying and assessing cardiovascular function in many of the mouse mutants with the most deleterious cardiovascular phenotypes (55). The first noninvasive imaging of mouse fetuses in utero was carried out using a conventional 7.5-MHz transabdominal ultrasonography system, which largely entailed the use of spectral Doppler echocardiography to acquire quantitative hemodynamic data on mouse fetuses (12). Since then, several studies have followed showing the successful use of noninvasive Doppler echocardiography for evaluating fetal hemodynamic function in transgenic or knock-out mouse models (16, 46). However, the resolution in these ultrasound systems was generally inadequate for imaging structural details associated with the heart and great arteries. With the recent development of vascular transducers with higher frequencies for clinical ultrasound systems, and the availability of 40-MHz ultrahigh-frequency ultrasound systems, two-dimensional (2D) resolution has been significantly improved (10, 33, 44, 49, 55, 56). A limitation of ultrahigh-frequency systems at present is the lack of phased arrayed transducers and color flow imaging, making it more difficult to pinpoint abnormal Doppler presentations with accuracy. A further complication is the fact that blood in fetal mice is echogenic at ultrahigh frequencies (20).
Using a clinical ultrasound system together with the 15-MHz high-frequency transducer, we conducted a noninvasive ultrasound screen of mouse fetuses for cardiovascular effects.55This study utilized echoplanes similar to those used in human echocardiography, which are fixed relative to the heart. This strategy is high throughputs and effective in recovering fetuses (ENU)-induced mutations by genome scanning. Over the variety of extracardiac defects. The efficacy of this screen was sections. Using this imaging protocol, we scanned over 10,000 good concordance with parallel presentations of histological structures inferred from these orthogonal imaging planes show cardiac structure and function in the small hearts of fetal mice. Structures inferred from these orthogonal imaging planes show good concordance with parallel presentations of histological sections. Using this imaging protocol, we scanned over 10,000 fetuses and detected a wide range of cardiac defects and also a variety of extracardiac defects. The efficacy of this screen was further demonstrated with mapping of two of the ethylnitrosourea (ENU)-induced mutations by genome scanning. Over all, these studies show that this novel ultrasound imaging strategy is high throughput and effective in recovering fetuses with congenital cardiovascular and extracardiac defects and has the added advantage that it is more easily learned by nonclinicians.

MATERIAL AND METHODS

ENU mutagenesis and breeding of mutagenized mice. Mutagenesis was carried out with intraperitoneal injections of C57BL/6J males with fractionated doses of ENU (100 mg/kg body wt, 4 times at weekly intervals). After a period of 10 wk for the recovery of fertility, the mutagenized G0 males were mated to C57BL/6J female mice. The resulting G1 males were again mated to C57BL/6J females to generate G2 females. Four G2 females were backcrossed to their G1 father, and the resulting pregnant G2 females carrying the G3 fetuses were subject to ultrasound scanning. This breeding screen provides the means to recover recessive mutations that may cause congenital heart disease. Further information on the mutagenesis and breeding protocols can be found at http://pga.jsax.org/protocols.html. All of the animal studies have been approved by the National Heart, Lung, and Blood Institute (NHLBI) Animal Care and Use Committee.

Ultrasound imaging and Doppler echocardiography. An Acuson Sequoia C256 ultrasound system with a 15-MHz L8 linear phased array transducer was used for ultrasound interrogation of the mouse fetuses. Pregnant female mice were anesthetized with isoflurane anesthesia (1.5% isoflurane in medical air containing 21% oxygen) and laid supine with all legs taped to electrocardiogram (ECG) electrodes for heart rate monitoring (450–550 beats/min). Body temperature was monitored via a rectal thermometer and maintained at 36–38°C using a heating pad and lamp. Hair was removed from the abdomen, and prewarmed ultrasound gel was applied. Ultrasound scanning was conducted on fetuses in utero, using the mother’s bladder as a landmark, with fetuses on the left and right uterine horns labeled as L1,2,3,4 (left side) and R1,2,3,4 (right side) (21). Color flow and spectral Doppler imaging were obtained from E12.5 to E19.5, and M-mode from E14.5.

Multiple presets and postprocessing features were selected from the Acuson Sequoia algorithms using the 15-MHz phased array transducer. We used a Doppler gate of 1 mm and a wall filtering preset of 1 for the Acuson spectral display. 2D resolutions are 440 μm axial and 630 μm lateral, with the lowest temporal resolution at 30 Hz. The initial 2D screening of fetuses was carried out using the full 2D imaging screen. This is followed by increased magnification, first to 20-mm2 frame size acquired at 53 Hz, followed by further enlargement to view the heart at 6-mm2 frame size acquired at 198 Hz. Color flow was added to either of the latter magnified 2D imaging frames and were acquired using 11 or 38 Hz, respectively. The color pulse wave Doppler was acquired at a pulse repetition frequency of 7.0 MHz, with the Nyquist limit at which aliasing occurs set at 3.4 m/s. M-mode tracings were obtained at 1,000 frames/s run at 100 mm/s. The M-mode spectral display was enlarged to allow accurate measurements of these small hearts in a specially designed structured report on the Acuson.

 Necropsy and histological examination. Stillborn pups were retrieved and fixed in 10% buffered Formalin. Necropsy was performed to examine the heart, great vessels, and aortic arch arteries. For histology, the heart and surrounding vessels were paraffin embedded, sectioned, and stained with hematoxylin and eosin. Some specimens were processed and examined by episcopic fluorescence image capture (EFIC) (41).

Genome scan analysis. To map the ENU-induced mutation, G2 C57BL/6J (B6) females carrying the mutation of interest were intercrossed with C3H/HeJ (C3H) mice to generate B6/C3H hybrid offspring. These were then further intercrossed for phenotyping by echocardiography, followed by analysis via necropsy and histology. DNA collected from the affected fetuses was PCR amplified using primers for 48 B6/C3H polymorphic microsatellite markers. The resultant PCR products were pooled and separated by capillary electrophoresis on the Avant 3100 Genetic Analyzer (Applied Biosystems), and the data generated were analyzed using recombination interval haplotype analysis (28). With this method, DMA markers located near the ends of each mouse chromosome are used to demarcate intervals that are treated as haplotypes for the purpose of linkage analysis. The frequency with which recombinant haplotypes are found across the entire genome in the affected fetuses is tracked. The ENU-induced mutation is expected to lie in a chromosome interval that is nonrecombinant, i.e., consistently homozygous for the mutant B6 strain in most or all of the affected fetuses.

RESULTS

To improve the throughput of ultrasound scans for cardiovascular phenotyping of mouse fetuses in utero, we evaluated the use of orthogonal imaging planes fixed relative to the fetus’ vertebral column, head, and overall body outline, all structures readily visualized by ultrasound. In this manner, standard views were established encompassing the sagittal, frontal, and transverse imaging planes (Fig. 1A). While imaging in each of these planes, a 2D cine clip or color flow Doppler images were captured by angulating the transducer around the selected axis. In the 2D imaging mode, ventricular walls and ventricular septum can be identified, but the atria and walls of the great vessels cannot be distinguished due to their thin walls, especially in early embryonic stages. However, with the help of color flow Doppler mapping, the chambers of the inflow and outflow tracts and relative position of the great arteries can be delineated. In this manner, most of the major elements of the heart can be evaluated, including the great arteries, aortic arch, inferior vena cava, and ductus arteriosus (Table 1). Below we
provide examples of ultrasound images captured along these three orthogonal planes, showing the structures that can be evaluated in each imaging plane. We also provide a comparison with histological sections presented in similar views.

Sagittal views. Sagittal imaging planes are obtained when the vertebral column of the fetus is captured in the long-axis view, showing a typical fetal position (Fig. 1B). The fetus is then interrogated in the sagittal plane by angulating the transducer in small increments, while focusing on the heart and great vessels. 2D imaging in this plane together with color flow Doppler mapping of blood flow allows the visualization of the right inflow tract and outflow tract (Fig. 2). In this view, the crossing of the pulmonary trunk and ascending aorta also can be observed, which often is perturbed with malalignment of the great arteries or outflow tract septation defects. Although the valves cannot be visualized, color flow Doppler nevertheless facilitates the detection of retrograde vs. anterograde flow across the tricuspid and pulmonary valves. This imaging plane also can provide a longitudinal view of the ductus arteriosus, allowing color flow mapping for viewing normal right-to-left anterograde flow or abnormal left-to-right retrograde flow in the ductal arch.

Examples of images obtained in the sagittal view are shown in Fig. 2. The color flow image in Fig. 2A shows, in systole, the inferior vena cava in blue with blood flow toward the right atrium, while blood flow in the ascending aorta going posteriorly is in red. In diastole, the inferior vena cava is in red due to reverse flow from right atrial contraction, with right ventricle in blue from right atrial blood flow (Fig. 2B). These ultrasound views can be compared with the histological sections in Fig. 2, G and H. Also seen in this view is the right ventricular outflow tract, which is visualized by blood perfusing the pulmonary trunk (PA in Fig. 2C), seen as a blue stream extending posteriorly around the aorta cut in cross-sectional view (AO in Fig. 2C). This resembles a short-axis view often used in clinical ultrasound (similar to histological section in Fig. 2H). A longitudinal view of the pulmonary trunk also can be obtained showing connection of the ductus arteriosus and descending aorta in a “hockey stick” configuration (Fig. 2D; compare with histological section in Fig. 2J). In this view, the aortic arch appears as if emerging from the center of the heart with a circular shape like a “candy cane” (Fig. 2E). In more oblique sagittal plane, a long-axis view of the left ventricle also can be obtained (Fig. 2F; compare with histological section in Fig. 2J).

Frontal views. Frontal views are obtained when the fetus is seen in its long axis, and the body and head are symmetrical (Fig. 1D). In this view, the heart is sectioned in the frontal plane, and both ventricles and interventricular septum can be observed (Fig. 3A). Color flow mapping allows delineation of the left outflow tract and blood inflow into both ventricles and, together with spectral Doppler analysis, can detect regurgitant flows in the left outflow or inflow tract (also see Cardiovascular defects, below). A 2D ultrasound image in the frontal plane is shown in Fig. 3A, which compares favorably with the histological section shown in Fig. 3B, showing long, narrow,
Color flow mapping in this imaging plane allows visualization of the left outflow tract and egg-shaped left ventricle. Color flow mapping in this imaging plane allows visualization of the left outflow tract or aorta (red) together with cross-sectional view of the pulmonary artery (blue) (Fig. 3C). Also seen is blue color flow into the ventricles during diastole (Fig. 3E). Corresponding histological sections are shown in Fig. 3, D and F, with spectral Doppler tracings confirming the color flow mapping (Fig. 3, G–I). It is in this imaging plane that we manipulate the transducer to obtain M-mode images, an imaging modality that provides the most accurate quantitative measures of ventricular chamber dimensions and wall thicknesses. This is achieved by angulating the transducer from the initial frontal plane to position the sample volume perpendicular to the interventricular septum all the while maximizing ventricular diameter (Fig. 4).

**Transverse views.** The transverse imaging plane corresponds to a short-axis view of the fetus (Fig. 1C). This view is the least informative and most difficult to acquire, requiring a great deal of time for appropriate positioning of the transducer. Examples of 2D images in the transverse view are shown from base to apex in color flow images shown in Fig. 5, A, C, and E, and these are compared with corresponding histological views in Fig. 5, B, D, and F. It should be noted that color flow is especially important for mapping heart structures in this view, with spectral Doppler analysis needed for further confirmation of the position of inflow vs. outflow (Fig. 5, G and H). In Fig. 5H, the spectral Doppler suggests an inflow pattern for the color flow seen in Fig. 5E. The spectral Doppler pattern for the outflow in Fig. 5C is shown in Fig. 5G. In this view, is not possible to obtain spectral Doppler tracings with maximal velocity, as the imaging plane is perpendicular to the great arteries.

**Ultrasound detection of developmental defects.** Using this body axes-guided ultrasound screening strategy, we ultrasound scanned 10,091 G3 fetuses carried by 1,190 pregnant G2 mothers backcrossed to 348 G1 fathers derived from G0 males that were ENU mutagenized. This breeding scheme constitutes a recessive screen that is likely to yield mutations causing the most deleterious phenotypes. The ultrasound scanning was conducted mainly between E12.5 and E17.5. E12.5 is the earliest stage at which the ventricles are clearly distinguishable, while beyond E17.5, the larger-sized fetuses are sometimes too deep for ultrasound imaging. Usually, each fetus is scanned two or three times, with fetuses initially scanned at E13.5 and rescanned a second and third time at E15.5 and E17.5, respectively. For fetuses scanned initially at E12.5, follow-up scans were carried out at E14.5 and E16.5. When fetuses with interesting cardiac presentations were found, additional scans were often performed at other stages to track disease progression.

The initial ultrasound scan is conducted to visualize the entire body of the fetus, using either the sagittal or frontal imaging plane. This allows quick evaluation for extracardiac defects. Then we magnify the area comprising the heart, adding...
on color flow to assist in the evaluation of cardiac structure and hemodynamics. Our ultrasound assessments include evaluating the relative size of the ventricles and the great arteries, the vessel’s connection to the ventricles, the relative position of the great arteries and the arches, evidence of pericardial effusion, increased flow velocity or aliasing of regurgitant blood flow in the outflow or inflow tracts, and altered wave forms associated with the inferior vena cava, ductus venosus, umbilical artery,

Fig. 3. Ultrasound images using frontal views. Color flow mapping in this E16.5 fetus is shown with the imaging plane moving anterior to posterior (A, C, and E), and corresponding histological sections are presented in similar orientations (B, D, and F). A and B: 2D ultrasound imaging shows the top of the LV, the interventricular septum, and the RV outflow tract (RVOT). In the histology image in B, the LV can be seen in an egg-shaped configuration, while the RVOT is seen as a half moon. C and D: color flow imaging (C) delineates the AO in red and the cross section of the PA in blue. Similar anatomy is seen in the histological image (D). E and F: color flow (E) in blue depicts two ventricles, and this same view is seen in a corresponding histological section (F). G–I: spectral Doppler analysis was used to confirm the position of the outflow and inflow. Correlating with the color flow in C is the spectral Doppler in G that shows the maximal aortic velocity, while H shows a spectral Doppler for the PA. The inflow spectral Doppler in I was obtained from the blue color flow in E. The heart rate in this fetus was determined to be 230 beats/min (bpm).
or vein. If this initial scan suggests cardiovascular defects, such as enlargement of the heart, increased wall thickness, regurgitant flow or aliasing, then additional ultrasound scans in the same and other imaging planes are pursued for more detailed evaluation. We note that M-mode imaging, which provides the most accurate quantitative measurements of the heart, is only feasible on a limited number of fetuses, given that the position of the fetus often makes it time consuming to achieve the requisite imaging plane.

Particularly helpful in the identification of fetuses with structural heart defects is the detection of regurgitant flow by color flow Doppler analyses. If there is outflow regurgitation due to semilunar valve insufficiency, then placement of the sample volume in the regurgitant stream would show an outflow envelope in systole and an abnormal regurgitant envelope in diastole. Alternatively, if there is inflow valve regurgitation, then placement of the sample volume in the regurgitant flow would show normal E and A waves in diastole, indicating that the regurgitant flow is likely due to atrioventricular valve regurgitation during systole. In the event we observe a compound outflow envelope and inflow E and A wave tracings, we would angle the transducer slightly to acquire one or the other flow patterns.

In total, our screen identified developmental anomalies in 777 of 10,091 fetuses scanned (Table 2). These are broadly categorized as with cardiovascular defects, prenatal lethal, growth retardation, hydrops fetalis, craniofacial/head anomaly, and body wall defects (Table 2). These are overlapping categories, as fetuses with hydrops also may be found with cardiovascular anomaly and/or craniofacial anomaly, etc. (Table 2). Prenatal lethality was observed throughout the scanning period and is indicated by fetuses with no heart beat, which often is combined with hydrops fetalis. Hydrops is characterized by fluid around the skull and body and is observed as a halo of echotransparency around the fetus (Fig. 6A) or, in whole mount views, as fluid retention under the skin (Fig. 6B). Body wall defect is indicated when the liver, gut, and/or heart is seen protruding outside the body wall (Fig. 6, C and D). The fetus in Fig. 6D also shows an omphalocele and eye defect. Encephalocele is indicated when part of the brain is seen protruding outside the cranium, and often this is associated with polyhydramnios (Fig. 6, E and F). Not all of these indications are necessarily related to cardiovascular defects, but fetuses presenting with any of these defects are subject to a more detailed ultrasound examination for possible cardiovascular presentations.

Fig. 4. Analysis by M-mode imaging. An E16.5 fetus exhibits pericardial effusion (PE), seen as increased echolucency around the heart (white arrows in A). Hypertrophy is also indicated by the M-mode tracings (B–D), which showed ventricular wall movement through the cardiac cycle. Heart cavity and wall thicknesses were measured using the M-mode tracings in diastole (C) and systole (D). These measurements provide a calculated shortening fraction of 39% and an ejection fraction of 77%. The heart rate was determined to be 255 bpm. Note the LVPW is actually the left ventricular lateral wall, while the RVAW is the right ventricular lateral wall in this front view. LVPW, left ventricular posterior wall; IVS, interventricular septum; RVAW, right ventricular anterior wall; d, diastole; s, systole.
Fig. 5. Ultrasound imaging using transverse views. Ultrasound images in the transverse planes (A, C, and E) and comparable histological sections (B, D, and F) are presented from rostral to caudal in an E16.5 fetus. A and B: the PA crossing over the AO in cross section and bifurcating into 2 PA branches. C and D: the AO posterior to the PA. E: both ventricles by color inflow mapping in diastole. F: the corresponding histological image. Color flow mapping with spectral Doppler confirmation of outflow and inflow waveforms is presented in G and H. The heart rate for this fetus was determined to be 204 bpm. PV, pulmonary vein.
Cardiovascular defects. Cardiovascular defects were seen in 425 of 10,091 fetuses ultrasound screened. Given the limited 2D spatial resolution of ultrasound, diagnosis of the specific cardiac defects required further analysis by necropsy and histology of the fetuses. Our analyses showed phenotypes including persistence truncus arteriosus (PTA), transposition of the great arteries, double-outlet right ventricle, Tetralogy of Fallot, aortic stenosis, pulmonary stenosis, pulmonary atresia, various aortic arch anomalies, dextrocardia, atrial/ventricular septal defects, common atioventricular canal, hypertrophy, and ectopia cordis. Below we show the ultrasound presentations for two fetuses as examples of cardiovascular defects that are seen by noninvasive fetal ultrasound.

Shown in Fig. 7 is an E16.5 fetus with ultrasound presentations that indicated possible persistent truncus arteriosus with craniofacial/head defects. Frontal views from the ultrasound scans revealed a small and abnormally shaped head (Fig. 7A), which later was found through examination of the stillborn pup as resembling holoproencephaly (Fig. 7B). Ultrasound interrogation of this fetus indicated a single outflow vessel. This can be seen with color flow imaging in systole (Fig. 7C), and in diastole, we observe an abnormal high-velocity (0.55 m/s) regurgitation back into both ventricles (Fig. 7, D and E). These presentations would suggest PTA, which might be associated with dysplastic valves. Indeed, PTA was confirmed in the subsequent necropsy of the fetus, which died at birth (Fig. 7F).

Table 2. Frequency of developmental anomalies detected by fetal ultrasound

<table>
<thead>
<tr>
<th></th>
<th>No. of Families1</th>
<th>G2 Females2</th>
<th>Fetuses Screened3</th>
<th>Abnormal Fetuses4</th>
<th>With Cardiac Defects5</th>
</tr>
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<tbody>
<tr>
<td>Total screened</td>
<td>348</td>
<td>1,190</td>
<td>10,091</td>
<td>777</td>
<td>163 (47%)</td>
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<tr>
<td>Cardiovascular anomalies</td>
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<td>Prenatal lethality</td>
<td>148 (43%)</td>
<td>214 (18%)</td>
<td>324 (3.2%)</td>
<td>42 (42%)</td>
<td>87 (20%)</td>
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<tr>
<td>Hydrops fetalis</td>
<td>100 (29%)</td>
<td>130 (11%)</td>
<td>188 (1.8%)</td>
<td>24 (24%)</td>
<td>90 (21%)</td>
</tr>
<tr>
<td>Growth retarded</td>
<td>76 (22%)</td>
<td>93 (7.8%)</td>
<td>123 (1.2%)</td>
<td>16 (16%)</td>
<td>67 (16%)</td>
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<tr>
<td>Craniofacial/ head defects</td>
<td>16 (4.6%)</td>
<td>19 (1.6%)</td>
<td>22 (0.2%)</td>
<td>2 (2.8%)</td>
<td>8 (1.9%)</td>
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<tr>
<td>Abdominal wall defects</td>
<td>6 (1.7%)</td>
<td>7 (0.6%)</td>
<td>11 (0.1%)</td>
<td>1 (1.4%)</td>
<td>8 (1.9%)</td>
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1Families are defined by the G1 males used to generate the G2/G3 offspring. 2Nos. in parentheses represent percentage of all G2 females scanned that have fetuses exhibiting the indicated defects. 3Percentages represent percent of 777 abnormal fetuses with the indicated defects. 4Fetuses with cardiovascular defects in conjunction with other indicated defects. Nos. in parentheses represent percentage of fetuses with cardiac defects that also have the indicated defects. This is based on a total of 425 fetuses with cardiovascular anomalies.
Fig. 7. Ultrasound imaging shows head defects together with persistent truncus arteriosus. 2D imaging in the frontal plane of an E16.5 fetus shows a small and abnormal head (A). This pup died at birth and exhibited a phenotype resembling holoprosencephaly and bilateral clefts (B). Color flow mapping in the frontal plane showed a single, long, great vessel going toward the head in red (C), with regurgitant blue flow in diastole going back into the ventricle and mixing with the blue color inflow (D). This was associated with aliasing, which is indicated by a double asterisk (D). This regurgitant flow was confirmed by spectral Doppler (E), which showed a heart rate of 177 bpm. Necropsy (F) showed that the cardiovascular defect in this fetus was persistence truncus arteriosus (PTA), which histology confirmed as a single vessel (CT) with VSD (*). Other sections (not presented) show the CT giving rise to coronaries, AO, and PAs. CT, common trunk; OFT, outflow tract; R, regurgitant flow; VSD, ventricular septum defect.
Histology showed dysplastic valves as well as a common trunk overriding the ventricular septal defect and interventricular septum (Fig. 7, G and H).

Another fetus with cardiovascular abnormalities is shown in Figs. 8 and 9. This fetus was ultrasound interrogated in all three imaging planes at E16.5. In the frontal view, enlarged ventricles were observed with “torch”-like outflow tract coming from the right ventricle and regurgitant flow from the outflow tract to both ventricles (Fig. 8, A–D). To ascertain the connection between the great arteries and aortic arch, views from the sagittal plane were acquired. They show that the outflow tract is connected with the dorsal aorta, with severe regurgitant flow retrograde around the aortic arch (Fig. 8, E and F). This confirms that the vessel seen to arise from the right ventricle in the frontal view (Fig. 8B) is likely the aorta. Because these ultrasound interrogations did not provide any insight into the connection of the pulmonary artery, this fetus has either double-outlet right ventricle or transposition of the great arteries, with or without pulmonary atresia. This also could be another case of PTA. The sagittal ultrasound image of this fetus also showed holosystolic tricuspid regurgitation with systolic velocity over 1.0 m/s (Fig. 9, A and B). This tricuspid regurgitant flow is likely due to dysplastic atrioventricular valves or heart failure (11, 8). By gradually turning the transducer and focusing on the inflow valves, we could ascertain via a transverse view that the ventricles were enlarged, with regurgitant flow through both right-sided and left-sided atrioventricular valves (Fig. 9, C and D). These findings suggest a common atrioventricular canal. However, we have no confirming necropsy data, as this fetus died before birth.
Heritability of mouse mutations. Two of the mutant families recovered from the ultrasound screen were further interbred with C3H mice to examine heritability of the mutant phenotypes and to map the underlying ENU-induced mutation (55). One family referred to as family 220 exhibited cardiac defects and omphalocele (Fig. 6, C and D), and a second family, family 217, exhibited PTA and holoprosencephaly (Fig. 7). Breeding in both families showed that the mutant phenotypes were heritable as monogenic recessive mutations. Microsatellite markers polymorphic between C3H and B6 were used to track the mutation in the B6/C3H hybrid offspring. Because the mutations were originally generated in the B6 background, it is expected to track with the B6 microsatellite markers. Analysis of the genome scan data using recombinant interval haplotype analysis successfully mapped the mutation in both families (28) (Table 3). The mutation in family 220, associated with omphalocele and eye defect, was localized to a 74-Mb interval between the centromere and marker D17mit93 on chromosome 17. The mutation in family 217 was mapped to chromosome 2, in a 36-Mb interval between markers D2mit7 and D2mit38.

DISCUSSION

We show the efficacy of a novel fetal ultrasound imaging protocol for interrogating the fetal mouse heart using three orthogonal echocardiographic planes defined by the fetus’ vertebral column and body axes. This ultrasound imaging strategy is both high throughput and very effective in identifying mouse fetuses with a wide range of congenital cardiovascular defects. Using this screening protocol, a litter of 8 mouse fetuses can be scanned in 15 min, which extrapolates to ~10,000 fetuses annually, a throughput not achieved in this study for lack of mouse fetuses to screen. The noninvasive nature of the fetal ultrasound screen is advantageous, not only because it allows cardiovascular function to be assessed under physiological conditions but because the mother and her surviving pups need not be killed for the phenotyping. Moreover, because 20% of the fetuses with cardiovascular defects died before birth and many more died neonatally, prenatal fetal screening greatly improves the efficiency of our screen in recovering fetuses with heart defects. Our findings suggest that simply retrieving and analyzing dying fetuses would be much less productive, since only 20% of fetuses dying prenatally exhibited ultrasound presentations indicative of cardiovascular defects. When fetuses were found with extracardiac defects, we usually performed more extensive ultrasound interrogations, as clinical studies have shown that 17–62% of human fetuses with cardiac defects also have some extracardi-
gestation when real-time examination of the human fetal heart is currently limited (7, 8). Clinical centers specializing in fetal echocardiography treat color Doppler as an integral component of fetal cardiac evaluation, as it is well established that color Doppler allows easy detection and diagnosis of the majority of major fetal cardiac anomalies (1). In our screen, we routinely use color flow mapping together with aliasing to detect increased outflow velocity and/or regurgitant streams, presentations often associated with mouse fetuses with significant structural heart defects. Because the atrioventricular and semilunar valves are normally competent in the fetus, the regurgitations might reflect valvular insufficiencies arising from a primary valve defect (26, 36, 43, 48, 51) or they could stem from heart failure arising from a primary myocardial defect (32) or other structural heart defects (13, 15, 17, 29, 37). In the event of retrograde diastolic flow through the ductus arteriosus, a spectrum of right heart obstructions might be indicated, such as pulmonary stenosis, or pulmonary atresia (24, 47), while retrograde flow in the ascending aorta might denote aortic stenosis and hypoplastic left heart syndrome (3, 18, 50).

Our screen does not address defects in heart development causing early embryonic lethality, since the poor 2D spatial resolution of clinical ultrasound systems restricts our screen to E12.5 and older fetuses. We also note that the specific diagnosis of structural heart defects cannot be made by our ultrasound interrogations alone but requires further detailed phenotyping by necropsy and histopathology. Because such studies are very time consuming, an important goal of our screen is to identify fetuses that are good candidates for such detailed phenotyping analyses, all the while minimizing time spent on interrogating normal fetuses. We note that >95% of the fetuses scanned show no obvious abnormal cardiac presentations. Thus we use ultrasound screening largely for rapid qualitative assessments to identify fetuses likely to have significant structural heart defects.

Our study showed that extracardiac defects also can be detected by ultrasound scanning, including craniofacial/head defects and body wall closure defects. We found 22 of 777 abnormal fetuses as having craniofacial/head defects and 11 with body wall closure defects. This contrasts with 425 showing cardiovascular defects. This lower incidence in noncardiac anomalies could be due to the limited 2D spatial resolution of the ultrasound scan. However, we note that omphalocele and gastroschisis are reported to occur in the human population at an incidence of 0.02–0.01% (53) and are similar to the incidences reported for encephalocele and holoprosencephaly. In contrast, congenital heart defects occur at a much higher rate of 0.5–5% of live births (14, 40). These differences in the incidences of cardiac vs. noncardiac defects in humans compare surprisingly favorably to the difference we observed for the incidence of heart defects vs. body wall closure defects and craniofacial/head defects in fetal mice.

We showed the heritability of mutation in two mutant mouse lines, family 217 with PTA and holoprosencephaly and family 220 with cardiac defects associated with omphalocele. We mapped the mutation in family 217 to chromosome 2 and in family 220 to chromosome 17. Perusal of the chromosome intervals did not suggest any obvious candidate genes based on known knockout mouse models or human mutations. For example, genes linked with holoprosencephaly, SIX3, ZIC2, Gli2, PATCHED-1, TGIF, and SHH, were not found on the mapped chromosome 2 interval in family 217 (6, 9, 25, 38, 39, 52). Thus these mutant models promise to uncover novel genes involved in congenital heart disease.

Overall, our study validates the use of orthogonal imaging planes guided by the fetus’ axial skeleton for noninvasive mouse fetal echocardiography. These standard views allow reliable comparison of ultrasound data between different fetuses and even between different studies. This novel ultrasound imaging strategy can be used not only for high-throughput screening but also for detailed phenotyping of heart defects in knockout and transgenic mouse models. Although, for the purpose of our screen, our ultrasound assessments were largely qualitative, quantitative assessments can be undertaken for more detailed phenotyping of specific mutant mouse models. As a practical matter, these orthogonal imaging planes are more easily learned by nonclinicians and hence could facilitate the incorporation of high-frequency ultrasound imaging for fetal mouse cardiovascular phenotyping in the research laboratory. We conclude that ultrasound phenotyping combined with ENU mutagenesis can facilitate the recovery of mutations causing congenital heart defects and yield new insights into the genetic basis for human congenital heart disease.

**ACKNOWLEDGMENTS**

We thank Barbara Knowles and Luanne Peters for access to the ENU mutagenesis mouse colony through the Guest Investigator Program of the

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**Table 3. Mutation mapped by recombinant haplotype analysis**

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*P, marker on proximal end of chromosome; D, marker on distal end of chromosome. †Recombinant haplotypes refer to no. of C57BL6/C3H mutant offspring heterozygous or homozygous for C3H microsatellite markers on the respective chromosomes. ‡No. 0 in bold indicates nonrecombinant haplotype intervals where mutation is likely situated.
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REFERENCES


