Noninvasive, In Utero Imaging of Mouse Embryonic Heart Development With 40-MHz Echocardiography

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Background—The increasing number of transgenic and targeted mutant mice with embryonic cardiac defects has resulted in the need for noninvasive techniques to examine cardiac structure and function in early mouse embryos. We report the first use of a novel 40-MHz ultrasound imaging system in the study of mouse cardiac development in utero.

Methods and Results—Transabdominal scans of mouse embryos staged between 8.5 and 13.5 days of gestation (E8.5 to E13.5) were obtained in anesthetized mice. Atrial and ventricular contractions could be discerned from E9.5, and changes in cardiac morphology were observed from E9.5 to E13.5. Hyperechoic streaming patterns delineated flow through the umbilical, vitelline, and other major blood vessels. Diastolic and systolic ventricular areas were determined by planimetry of the epicardial borders, and fractional area change was measured as an index of contractile function. Significant increases in ventricular size were documented at each stage between E10.5 and E13.5, and the ability to perform serial imaging studies over 3 days of embryonic development is described. Finally, the detection of vascular cell adhesion molecule 1 (VCAM-1) homozygous null mutant embryos demonstrates the first example of noninvasive, in utero analysis of cardiac structure and function in a targeted mouse mutant.

Conclusions—We used 40-MHz echocardiography to identify key elements of the early mouse embryonic cardiovascular system and for noninvasive dimensional analysis of developing cardiac ventricles. The ability to perform serial measurements and to detect mutant embryos with cardiac defects highlights the usefulness of the technique for investigating normal and abnormal cardiovascular development. (Circulation. 1998;98:912-918.)

Key Words: ultrasonics ■ echocardiography ■ imaging ■ morphogenesis

The large number of mouse models with congenital heart defects, many of which result in early embryonic lethality, has resulted in a need for new technologies that allow the study of cardiac form and function in utero in mouse embryos. Previous investigations have studied fetal mouse cardiac anatomy and vasculature by examination of fixed specimens by histology, scanning electron microscopy, or magnetic resonance microscopy. None of these methods have provided real-time imaging of live embryos. More recent studies have looked at mouse embryonic cardiac function with video microscopy, which requires surgical exposure of the embryos and is therefore unsuited to serial measurements. Transabdominal 7.5-MHz Doppler has been used to make noninvasive measurements of embryonic heart but was limited in resolving anatomic details, particularly at earlier embryonic stages. Echocardiography in the frequency range of 3 to 7.5 MHz is an established method for evaluating fetal cardiac anatomy and function in humans beyond the first trimester and, with transvaginal techniques, as early as late first trimester. A higher-resolution, real-time ultrasound imaging technique would be invaluable in the study of mutant mouse phenotypes, especially during the early stages of cardiac morphogenesis.

High-frequency (40- to 50-MHz) ultrasound imaging, referred to as UBM, allows high-resolution in utero imaging of live mouse embryos. For this study, 40-MHz UBM was used to image the developing hearts of live mouse embryos in utero from gestational age 8.5 to 13.5 days (E8.5 to E13.5). We demonstrate 40-MHz echocardiographic images of the early embryonic mouse heart, allowing quantitative dimensional analysis of the cardiac chambers and serial studies over several days of development. In addition, we demonstrate the first noninvasive, in utero detection of embryos homozygous for a single gene mutation (VCAM-1) resulting in abnormal cardiac development and embryonic lethality. The ability to monitor and follow cardiac development in utero by this approach should be of great benefit in studying form and function relationships in mouse models of cardiovascular disease.

Methods

Animals

All animals used in these studies were maintained according to protocols approved by the Institutional Animal Research and Care Committee at New York University Medical Center. Timed pregnant...
CD1 mice (Charles River, Kingston, NY) and mice heterozygous for a null mutation of VCAM-1\textsuperscript{12,13} were anesthetized with sodium pentobarbital (5 mg/100 g body wt IP) with added magnesium sulfate (MgSO\textsubscript{4} · 7H\textsubscript{2}O, 10 mg/100 g body wt). Magnesium, a known tocolytic agent, was added to decrease the spontaneous uterine contractions that interfere with image acquisition. Although both agents can have cardiac depressant actions that may introduce confounding factors with functional assessments, nevertheless, anesthesia is required to enable the acquisition of reproducible data. In staging the embryos, day 0.5 (E0.5) was defined as 12 noon of the day a vaginal plug was found after overnight mating. Once the mouse was anesthetized, its lower abdomen and back were wet-shaved to provide a clear window to the embryos, because hair and the trapped microbubbles of air in the hair present an impenetrable barrier to ultrasound at these high frequencies.

The mouse was then placed on the lower level of a 2-level stage, and a small water bath was fitted onto the skin to provide a coupling medium for the transducer (Figure 1A and 1B). The stage holding the mouse was a simple wooden block (L × W × H = 85 × 120 × 40 mm) with a trough cut out (W × H = 40 × 15 mm) in which the anesthetized mouse was laid. The water bath was formed by either a 25-mm-diameter plastic cup or a Petri dish with a 25-mm-diameter hole in the center pinned to the top level of the stage to decrease the chances of extrinsic compression from a tight bath. As has been reported previously,\textsuperscript{7} we noted sensitivity of the embryos to temperature and attempted to maintain normal maternal temperature with external heat lamps.

**Image Acquisition**

A high-frequency (40- to 100-MHz) UBM system was developed and constructed in our laboratory\textsuperscript{14} and was previously demonstrated to be useful for in utero imaging of live mouse embryos\textsuperscript{7} (Figure 1C). For the present study, a focused 40-MHz transducer was used, with measured lateral resolution of 90 \(\mu\text{m} \), axial resolution of 30 \(\mu\text{m} \), and depth of penetration of 7 to 10 mm.\textsuperscript{14} The mechanical system scans over an 8-mm linear path, producing real-time images at a rate of 8 images per second, which are then output as video to a monitor/VCR for viewing and capture. Precise and repeatable control over the position of the two-dimensional image plane was made possible with a small motion stage and stepper motor, allowing the image plane to be moved across the embryo in increments ranging between 10 and 100 \(\mu\text{m} \).

Imaging was started from one flank, and embryos were sequentially imaged across the lower abdomen to the opposite flank. Embryos situated too deep in the abdomen were scanned to document their presence but were excluded from data analysis because of poor resolution. The ability to scan adjacent embryos helped in tracking embryos; however, in case of doubt, the embryos were skipped to avoid double sampling, especially when associated with excessive spontaneous uterine movements. Each litter had 6 to 10 embryos available for imaging (average litter size of CD1 mice is 10 to 12 embryos; VCAM-1 mutant mice typically have smaller litters, 5 to 8 embryos). After initial localization of an embryo and optimal placement of the water bath, sequential transabdominal linear scans were obtained at increments of 20 or 100 \(\mu\text{m} \), depending on the size of the embryo and the region of interest, thus imaging the entire embryo. Scan planes were modified by changing the orientation of the mouse with respect to the scan plane, and images were obtained in 2 orthogonal planes for each embryo. An effort was made to obtain views approximating the transverse, frontal, or sagittal planes but was sometimes limited to oblique planes by the position of the uterus in the abdomen. A total of 234 embryos staged between E8.5 and E13.5 were imaged with UBM over the course of this study.

Toward the end of the present imaging study, a UBM image-guided 40-MHz continuous-wave Doppler system was developed\textsuperscript{15,15a} and used to obtain nonambiguous in utero heart rate data by measuring waveforms of the umbilical artery and vein in 10 E13.5 embryos from 2 different litters (Figure 2).

**Image Analysis**

All imaging sessions were recorded in S-VHS video format that allowed off-line review and analysis. Although cardiac activity was discernible as early as E8.5, the heart structures at E8.5 to E9.5 were too small for reliable cardiac measurements to be made. In the older embryos (E10.5 onward), scans through the embryonic heart were selected and off-line analysis was performed with a Hewlett Packard Sonos 1000 clinical ultrasound system. At E10.5 and E11.5, embryos with scans in the transverse/oblique transverse plane showing a small part of the atria and a view of both ventricles were selected (Figure 3A through 3C). At E12.5 and E13.5, views representative of the 4-chamber view were selected (Figure 3D through 3F). Calibration data (4-chamber view were selected (Figure 3D through 3F). Calibration
of measurement software was performed by use of scale markers on the recorded video screen. Sequential video fields were analyzed, and frames representative of ventricular end systole and end diastole were determined. Given the echogenicity of the blood pool, the endocardial borders could not be identified; hence, the ventricular area was determined by planimetry of the epicardial borders (Figure 3A and 3D) similar to previous measurements made with video microscopy on surgically exposed mouse embryos.12,13 Because of the limitations of the relatively low frame rate in reliably identifying end diastole and end systole, an average of the maximal and minimal areas obtained for 4 cardiac cycles was taken to be representative. At E12.5 and E13.5, areas were determined for RVs and LVs individually, whereas combined ventricular area was determined at E10.5 and E11.5 because of the lack of significant development of the interventricular septum. The FAC was calculated for each of these as the difference between the diastolic area and systolic area, indexed as a percentage of diastolic area.

Data are reported as mean±SD. To test for intraobserver variability, 10 embryos from E12.5 or E13.5 and 7 embryos from E10.5 or E11.5 were analyzed. Four diastolic and 4 systolic frames were selected for each embryo, and ventricular areas were determined. Variability was expressed as difference from the mean of the 2 results in percentage of the mean for diastolic area, systolic area, and averaged FAC. The independent t test was used to compare ventricular areas between the groups.

Results

UBM Images of Normal E8.5 to E13.5 Mouse Embryos

Images obtained by 40-MHz echocardiography had some characteristics that differ from lower-frequency clinical ultrasound.

At this high frequency, the blood pool appears highly echogenic, and as such, the heart was readily identified on real-time images as a bright, highly echogenic structure with rhythmic contractions (Figure 3). This also imparted a dramatic speckle movement due to blood flow, which on real time made the blood vessels prominent. This effect was less apparent on still frames, in which the contrast from the moving speckle pattern was lost. On real-time video images, the umbilical vessels (Figure 2A), vitelline vessels, dorsal aorta (Figure 1C), great veins, and cerebral vessels were all easily identified.

As with clinical fetal ultrasound, embryonic orientation was first determined by scanning from top to bottom. The consistent orientation of normal mouse embryos within the uterus, with the placenta and the tail to the right of the embryo, was confirmed and used to identify cardiac structures. Cardiac contractions were identified in some E8.5 embryos, and by E9.5 the common atrium and ventricle could be distinguished. By E10.5 to E11.5, the distinction between the presumptive LVs and RVs became more apparent. The outflow tract was prominent (Figure 4A through 4C), and 2 parallel streams of blood flow were apparent on real time. Synchronous and discernible atrial and ventricular contractions were apparent as early as E9.5, with atrial con traction occurring at end diastole. At E12.5 to E13.5, there was a marked increase in the size of all 4 chambers of the heart, and a change in the configuration of the outflow tract was apparent (Figure 4D, E13.5). By sequential scanning, volumetric structures could be followed through consecutive
image planes (Figure 5). In stage E12.5 and E13.5 embryos, a distinct "C"-shaped aorta could be traced, arising from the LV and passing to the right of the pulmonary artery before looping to the left (Figure 5C). The placenta changed from a discoid structure at E10.5 to a larger planoconvex structure by E13.5, with prominent sinusoids on its outer surface.

Embryos have previously been imaged over 3 consecutive days, after which the pregnancies went to term and no adverse effects on live-born animals were observed. Although the objective of the present study was to demonstrate the detailed anatomic data on embryonic cardiac morphology possible with 40-MHz echocardiography, we recognize the importance of the technique for serial studies of individual embryos, particularly in studies of mutant embryos to follow the dynamic progression of specific cardiac defects. To further test the feasibility of serial studies, 4 litters were imaged on 3 consecutive days, from embryonic stage E11.5 to E13.5. After 1 additional day (E14.5), the anesthetized mothers were killed by cervical dislocation and the embryos dissected and examined under a dissection microscope. In all embryos in the 4 litters (n=31), both the UBM images and gross morphology were normal.

Ventricular Dimensions and FAC

Ventricular area was obtained by planimetry of the epicardial surfaces in a group of embryos (82 of 147 normal embryos staged between E10.5 and E13.5) selected to have the best views for performing quantitative analysis (see the Methods section). There was a significant increase in ventricular size with age. From E10.5 to E11.5, the combined ventricular area increased from 1.47±0.33 to 2.02±0.21 mm² in diastole and from 0.86±0.18 to 1.27±0.15 mm² in systole (P<0.001). Similar significant increases were noted from E12.5 to E13.5 for both ventricles (Table 1). At these stages, the RV and LV were well balanced, with no significant difference in size. The FAC was 41±7% at E10.5 (n=15) and 37±5% at E11.5 (n=16). The FAC was similar for both ventricles through E12.5 (RV, 32±7%; LV, 34±5%; n=21) and E13.5 (RV, 34±6%; LV, 34±6%; n=30) (Table 1). Because combined ventricular area was used at the earlier stages, no direct comparison was made with older embryos. At E13.5, heart rate measured by 40-MHz Doppler was 196±27 bpm (Figure 2), which is similar to reported values of 157±27 bpm and 173±7.5 bpm for this gestational age.

The intraobserver variabilities at E12.5 and 13.5 were 0.4±2.3% for RV diastolic area, 1±1.6% for RV systolic area, 1.1±2.7% for RV FAC, 0.8±1.1% for LV diastolic area, 0.2±2.1% for LV systolic area, and 1.8±3.6% for LV FAC. Corresponding values at E10.5 and E11.5 were 0.6±2.9% for combined diastolic area, 0.6±0.7% for systolic area, and 0.7±7.3% for FAC. To study the reproducibility of data in different litters and the variability of the technique, results from 2 litters at E13.5 were compared (Table 2) and showed no significant differences.

Abnormal embryos observed during the course of this study included numerous dead, resorbing embryos, which are expected and found in most strains of mice. Two E13.5 embryos were noted that showed features of hydrops, with evidence of pleural and large pericardial effusions and a small, sluggishly contracting heart. In 1 of these animals, the calculated RV FAC was 19% and the LV FAC was 13%.

VCAM-1 Mutant Studies

To investigate the utility of 40-MHz echocardiography in the noninvasive detection of targeted mouse mutant embryos with defects in cardiac development, we performed in utero imaging studies on 4 litters (n=29) from VCAM-1 heterozy-
gous intercrosses. VCAM-1 homozygous null mutant embryos have been reported to die in utero, exhibiting 2 distinct phenotypes. Approximately half of the null mutants die before E11.5 and exhibit a failure of the allantois to fuse with the chorion. The remaining VCAM-1 null mutants appear to form a normal connection between allantois and chorion but still die at E12.5 from cardiac-specific defects, including a lack of epicardium and reduction of the ventricular myocardium and intraventricular septum.

Two VCAM-1 heterozygous mutant females were imaged at E10.5, after which the mice were killed and their embryos dissected for analysis. In the first mouse, 5 embryos were imaged on the left uterine horn, 1 of which was identified as a mutant from the prominent pericardial effusion, separation of allantois from chorion, and little or no cardiac activity (Figure 6A; compare with sagittal image of normal E10.5 embryo, Figure 1C). After dissection, the embryo showed the same gross morphological features as identified on ultrasound (Figure 6B) and was confirmed by Southern blot analysis of yolk sac tissue to be a homozygous mutant. In a second mouse, 9 embryos were imaged and 2 identified as VCAM-1 null mutants, subsequently confirmed by genotype analysis, with UBM features similar to those noted above. The ventricular area measured in the VCAM-1 homozygous mutant embryos (0.62 and 0.49 mm²) was well below that measured in normal E10.5 embryos (diastolic area, 1.47 mm²; systolic area, 0.86 mm²), and there was no discernible difference between diastole and systole in the detected mutants. Combined ventricular FAC was measured in 4 heterozygous VCAM-1 mutants in the same litter and was found to have no significant difference from FAC measured in normal E10.5 embryos (47±8% for VCAM-1 heterozygotes versus 41±7% for normal embryos). Interestingly, a third homozygous mutant was present in the litter that had normal appearance on UBM images and also appeared normal when examined for gross morphology.

A third mouse had 8 embryos, which all appeared normal when imaged on 2 successive days (E10.5 and E11.5), and subsequent genotype analysis showed that there were no homozygous mutants in the litter. Finally, a fourth mouse had 7 embryos, which were imaged at E12.5, 2 of which were obviously dead and resorbing, with a characteristic echogenic rim around their hearts. Both dead embryos were subsequently confirmed by genotype analysis to be VCAM-1 homozygous mutants.

**Discussion**

This study demonstrates the feasibility of noninvasive real-time imaging of the early embryonic mouse heart in vivo by high-frequency ultrasound. For the first time, dimensional analysis of the cardiac ventricles has been...
demonstrated with 40-MHz echocardiography, and ventricular area change has been measured as a noninvasive index of contractile function. The developmental stages studied, E8.5 to E13.5, correspond to ~3 to 6 weeks of human gestation and are the earliest hearts to be studied in utero in the developing mouse. Gui et al. used transabdominal 7.5-MHz Doppler ultrasound to obtain functional data from E10.5 to E19 mouse embryos, but the frequency used was inadequate to image embryonic cardiac anatomy, and therefore positioning of the Doppler sample volume was only inferred from the similarity of the waveforms to those measured in later-stage human fetuses. The future combination of Doppler measurements, guided by UBM imaging to specific regions of the developing heart, should provide a powerful method for assessing cardiac function in mouse embryos. In this report, we provide preliminary data to show the feasibility of in utero UBM-guided Doppler measurements of umbilical blood velocity in E13.5 mouse embryos.

Video microscopy has been used previously to image the heart in chick, rat, and mouse embryos. Unlike chick embryos, however, mammalian embryos must be exposed by opening the maternal abdominal wall and uterus, or in the case of early-stage embryos (E8.5 to E10.5), by maintaining the embryos in culture, to allow optical imaging. In either case, viable embryos can be maintained only for short periods of time, precluding the possibility of serial studies over several days of development. MR microscopy of embryonic mouse heart gives excellent resolution, but noninvasive live cardiac MR imaging has not been demonstrated. The morphological changes in the developing mouse heart as determined by 40-MHz echocardiography are similar to those described previously by scanning electron microscopy and histological sections. There are currently no competing in vivo imaging methods that combine the image frame rate, resolution, and penetration of UBM for embryonic imaging and serial in utero studies.

Ventricular epicardial area measures have previously been correlated with ventricular mass and stroke volume in chick embryos. With UBM, we obtained ventricular epicardial areas and documented a significant increase in ventricular size with gestational age, as has been shown by more invasive methods. Given the constant remodeling of the embryonic heart during cardiac morphogenesis, calculation of ventricular volumes by use of geometric assumptions is likely to be inaccurate; hence, we used FAC as an index of contractile function. Moreover, during the earlier embryonic stages (E10.5 to E11.5), although a prominent interventricular sulcus is seen, the interventricular septum is poorly formed, and combined ventricular area change was taken to be a more reliable estimate of function at these stages. Ventricular areas measured by UBM at E12.5 to E13.5 are comparable to those reported by Keller et al., although our values for ventricular areas were somewhat larger (eg, E13.5 LV/RV diastolic area, 1.51±0.24/1.47±0.2 mm² in the present study versus 0.96±0.05/1.19±0.04 mm² in Keller et al.). However, the 2 studies differ in the methodology (noninvasive versus surgical exposure of embryos), the projections used to

obtain ventricular areas, and the strains of mice imaged, all of which could contribute to the differences noted.

One limitation of the current UBM imaging system is the relatively slow frame rate (8 images per second). Given a heart rate of 120 to 200 bpm, this gives only 2 to 4 frames for each cardiac cycle. We attempted to minimize the error by averaging 4 measures of apparent end systole and end diastole. The rapid heart rates and the movements associated with maternal respiration made it difficult to calculate heart rate visually from the video images. However, the heart rates that we measured by Doppler were similar to or even higher than those reported by other investigators at similar embryonic stages. We noted a sensitivity of the embryonic heart rates to the level of sedation and temperature and the importance of minimizing heat loss.

The availability of UBM-guided Doppler, currently being developed in our laboratory, will make it possible to monitor heart rate and blood flow patterns while obtaining real-time images. The marked prominence of the blood vessels, especially the umbilical vessels, which are isolated from the rest of the embryo, makes the use of image-guided Doppler interrogation an exciting possibility that would aid in establishing functional and morphological correlation in the developing mammalian cardiovascular system.

Ultimately, the value of the UBM imaging technology will be revealed in future investigations of mutant mouse embryos with cardiac defects. In the present study, VCAM-1 homozygous mutants were identified in utero by 40-MHz echocardiographic imaging. This is the first demonstration of the utility of UBM, or any other imaging method, to noninvasively detect targeted mutant mouse embryos with cardiac defects. Analysis of a limited number of VCAM-1 heterozygous mutants demonstrated normal values of ventricular FAC, providing further support for the hypothesis that these animals do not have impaired cardiac function. Future measurements of umbilical and cardiac Doppler waveforms in VCAM-1 mutant embryos should clarify whether the cardiac-specific phenotype is a secondary effect of abnormal placental circulation or is a primary defect associated with the loss of VCAM-1 expression. Serial studies of normal and mutant embryos with cardiac defects provide a unique method to determine the effects of specific genes on the functional development of the cardiovascular system.

Acknowledgments

This research was supported by a grant from the Whitaker Foundation. Dr. Turnbull is an Investigator of the American Heart Association/New York City Affiliate.

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_Circulation_. 1998;98:912-918
doi: 10.1161/01.CIR.98.9.912

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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