Ultrasound biomicroscopy-Doppler in mouse cardiovascular development

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Phoon, Colin K. L., and Daniel H. Turnbull. Ultrasound biomicroscopy-Doppler in mouse cardiovascular development. Physiol Genomics 14: 3–15, 2003; 10.1152/physiolgenomics.00008.2003.—The ability to modify the mouse genome has yielded new insights into the genetic control of mammalian cardiovascular development. However, it is far less understood how genetic factors and their consequent structural changes alter cardiovascular function, a void largely due to the lack of effective noninvasive techniques to assess function in the developing mouse cardiovascular system. In this review, we discuss the recent advances in ultrasound biomicroscopy (UBM)-Doppler echocardiography for analyzing cardiovascular function in the embryonic mouse in utero. “Cardiovascular function” encompasses broad aspects of physiology, including systolic and diastolic cardiac function, distribution of blood flow among various embryonic vascular beds, and vascular bed properties (impedance). A wide range of physiological measurements is possible using UBM-Doppler, but it is clear that the limitations of any single measurement warrant a multi-parameter approach to characterizing cardiovascular function. We further discuss the prospects for UBM-Doppler analysis of alternative vertebrate systems increasingly studied in developmental biology. The ability to correlate cardiovascular physiological phenotypes with their corresponding genotypes should lead to the elucidation of mechanisms underlying normal development, as well as embryonic disease and death.

cardiac morphogenesis; echocardiography; embryogenesis

THE ABILITY TO GENERATE transgene insertions and targeted mutations in the mouse has led to new insights into the genetic control of mammalian cardiovascular development. Among the earliest lessons from mouse knockouts, most striking was the realization that the cardiovascular system is almost uniquely critical for embryonic survival: major defects in virtually all other organ and body systems, with the exception of the hematopoietic system, do not cause lethality in utero (4). Over the past decade, a number of genetic factors and mechanisms underlying cardiac development have been elucidated (e.g., reviewed in Refs. 2 and 36), leading to increasingly accurate mouse models of human congenital heart disease (e.g., reviewed in Refs. 5 and 8). Far less understood are the precise roles of these genetic factors in altering cardiovascular function, which ultimately causes disease and death. This void is largely due to the lack of effective noninvasive methods to assess function in the developing mouse cardiovascular system, which also presents a major limitation in the analysis of disease progression in mouse models.

In this review, we discuss the recent advances in ultrasound biomicroscopy (UBM)-Doppler echocardiography for analyzing cardiovascular function in the embryonic mouse, in utero, describing the range of physiological measurements that are possible using this approach. Particularly in the mouse, UBM-Doppler has distinct advantages over other imaging modalities, which will be discussed briefly to place the role of UBM-Doppler in proper context. We further discuss the prospects for using UBM-Doppler in alternative vertebrate systems.
vertebrate systems commonly used for the study of cardiac development.

**BASIC PHYSIOLOGY OF THE EMBRYONIC CARDIOVASCULAR SYSTEM**

The embryonic mammalian cardiovascular system comprises the developing heart together with a number of interdependent, complex, and rapidly changing vascular networks, both within the embryo proper and in the extra-embryonic tissues that support the embryo during normal in utero development (Fig. 1). In its most basic form, the cardiovascular system can be viewed as being composed of three major components: 1) the embryonic circulatory system, made up of the heart, and the embryonic arteries and veins, of which the aorta and cardinal vein are the main branches; 2) the yolk sac circulation, made up of the vitelline arteries and veins; and 3) the allantoic or umbilical circulation, of which the allantoic artery and vein are the main branches.

Proper development and coordination of all three circulatory systems are critical for normal cardiovascular function and embryonic survival. Indeed, analyses of a large number of mouse mutants indicate that in utero lethality occurs in three distinct waves, corresponding to defects in the yolk sac (first wave), umbilical (second wave), and embryonic (third wave) circulatory systems (4). Therefore, any comprehensive approach to study functional development of the mouse cardiovascular system should provide the ability to measure physiological parameters in all three of the developing circulatory systems over a wide range of embryonic stages.

**ANALYSIS OF EMBRYONIC CARDIOVASCULAR FUNCTION: PREVIOUS TECHNIQUES**

Analyzing cardiovascular function in the mouse embryo remains challenging, and relatively few successes have been reported (for a review, see Ref. 32). Most “classic” studies in developmental physiology have employed either the servo-null micro-pressure system, refined by Clark and Hu (3), or implanted 20-MHz pulsed Doppler ultrasound sensors, originally developed by Hartley and Cole (14). Although this latter approach has recently been applied to surgically exposed mouse embryos (26, 27), most of the previous studies were done in the chick, because of easy access to the embryonic structures (for review, see Ref. 32). Such studies, however, are highly invasive and require significant perturbation and manipulation of the developing embryo. Several other imaging modalities are currently available, including high-speed videomicroscopy of surgically exposed mouse embryos (see Refs. 7, 26, 41); time-lapse videomicroscopy of cultured mouse embryos (see Ref. 24); magnetic resonance imaging of fixed mouse embryos (see Refs. 37, 38); high-speed fluorescent confocal videomicroscopy of zebrafish (see Ref. 17); and optical coherence tomography, recently applied to chick embryos (see Ref. 50).

A number of echocardiographic techniques have been developed to study hemodynamics in the adult mouse, largely derived from human clinical ultrasound (e.g., Refs. 16, 42, 49). However, spatial resolution in human echocardiographic systems, limited to 300–500 µm at best, is marginal even for adult mice and is inadequate for mouse embryos. The few studies that have utilized human echocardiography systems to examine mouse embryos have used real-time ultrasound images simply to locate the embryos in an anesthetized pregnant mouse, but have relied on Doppler ultrasound to obtain blood velocity waveforms as the basis for physiological analyses (13, 21, 40). In late gestation mouse fetuses, it is possible to identify separate structures such as the heart, aorta, umbilical vessels, and intracranial vessels and obtain Doppler signals from these different structures (28), but these stages are well beyond embryonic cardiac development. For the study of mouse models of normal and abnormal cardiovascular develop-

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**Fig. 1.** Schematic of the embryonic mammalian cardiovascular system. The three major compartments are the embryonic circulatory system, which includes the heart and intra-embryonic vascular bed (ACV, anterior cardinal vein; AoA, aortic arches; DAo, dorsal aorta; HV, hepatic veins; ICA, internal carotid artery; PCV, posterior cardinal vein); the yolk sac or vitelline circulation (VA, vitelline artery; VV, vitelline vein); and the allantoic or umbilical circulation (AlA = allantoic, or umbilical, artery; AlV = allantoic, or umbilical, vein).
ment, a noninvasive high-resolution echocardiography system would seem to provide an ideal approach to obtaining physiological data (1).

UBM-DOPPLER: A NONINVASIVE, IN UTERO APPROACH TO MEASURE CARDIOVASCULAR FUNCTION

UBM systems, employing 40-MHz ultrasound to achieve spatial resolutions of 30 μm axial and 90 μm lateral, have been developed in our laboratory to image the embryonic mouse heart and cardiovascular system noninvasively (33, 39, 43, 44; reviewed in Ref. 45). UBM analysis of cardiovascular function in mouse embryos has been further extended by including 40–50 MHz pulsed Doppler, enabling precise UBM image-guided Doppler measurements of blood velocity waveforms (Fig. 2; Refs. 1, 23, 30). Recently, commercial instrumentation, employing 19 to 55 MHz ultrasound (and with corresponding axial and lateral resolution as good as 28 μm and 62 μm, respectively) has also become available to perform similar UBM-Doppler analysis in the developing and maturing mouse cardiovascular system (10, 51).

UBM-Doppler differs from conventional clinical echocardiography systems in the use of high-frequency ultrasound, which permits higher spatial resolution, although with poorer depth of penetration. The higher-frequency Doppler transducers also facilitate acquisition of low-velocity blood flow signals. The two-dimensional UBM image is generated by mechanical motion of the imaging transducer (Fig. 2), so that the temporal resolution (frame rate) is limited by the sweep speed of the transducer motion, typically 8–10 frames/s (33, 51). The pulsed-wave Doppler beam allows a specific small area (sample volume) to be interrogated; this sample volume is guided by the UBM image itself (1, 30, 51). The Doppler beam should be aligned with the direction of blood flow as closely as possible, since the Doppler frequency shift and subsequent calculations of flow velocity vary as the cosine of the angle between the flow direction and the Doppler beam. Mouse embryos may be imaged through the maternal abdomen (10, 30, 39, 51) or “semi-invasively,” whereby the embryos are pulled out through a small abdominal incision but maintained in utero (23, 33). Because of the embryo’s extreme sensitivity to environmental perturbations and temperature, close thermoregulation of the entire maternal-embryonal unit is critical to obtaining physiological data (23, 30).

UBM-Doppler provides a critical advantage over lower frequency (human) echocardiographic systems in its ability to interrogate separate circulatory beds in the developing mouse embryo (10, 30, 39, 51) or “semi-invasively,” whereby the embryos are pulled out through a small abdominal incision but maintained in utero (23, 33). As described below, UBM-Doppler has been used to investigate parameters of cardiovascular function in both intra- and extra-embryonic circulatory systems from early embryonic day 8.0 (E8.0) through E14.5 and in mouse fetuses through the end of gestation.

Intracardiac UBM-Doppler

UBM has been used to image the mouse heart from embryonic (Fig. 3; Refs. 23, 39, 43, 44, 51) to neonatal stages (9, 29, 51). This has enabled noninvasive, in vivo assessment of function using indices derived from dimensional analyses of the cardiac chambers (see below). UBM has also been used to guide 40-MHz Dopp-
Flow in Early Embryonic Heart

Dorsal Aorta: A Window to Embryonic Cardiovascular Function

The dorsal aorta is easily identified with UBM from approximately E9.5 onward and can be interrogated with UBM-Doppler to measure aortic blood flow velocity (Fig. 4; Refs. 1, 23, 30, 33). As the primary artery in the developing mouse embryo, analysis of Doppler waveforms from the dorsal aorta has provided the basis for our characterization of blood flow (33) and cardiac systolic function in normal mouse embryos (30) and, more recently, in mutant mouse embryos with defined morphological defects in cardiac development (31, 34).

Extra-Embryonic Circulations: Umbilical and Yolk Sac Blood Flow

From our earliest imaging studies in mouse embryos, the umbilical vessels were recognized as providing an easily identifiable extra-embryonic circulatory system that can be studied with UBM-Doppler (Fig. 5; Refs. 1, 30, 39). Yolk sac vessels can also be visualized on UBM at most embryonic stages (23, 39), and we have recently determined that they are readily interrogated with Doppler, although no systematic study of vitelline blood flow characteristics has been performed at this point (Fig. 5).

Secondary Embryonic Circulatory Systems: Cerebral Blood Flow

Moving up the vascular tree from the major embryonic and extra-embryonic circulatory systems, we have also acquired Doppler waveforms from cerebral arterial vessels. The major cerebral arteries are evident on UBM as a result of the moving speckle patterns, and UBM-Doppler interrogation has yielded clear blood velocity waveforms, indicating that it should be possible to investigate cerebral blood flow in developing mouse embryos (Fig. 6).

UBM-DOPPLER CHARACTERIZATION OF CARDIOVASCULAR FUNCTION

Cardiac output, perhaps the most fundamental measure of cardiovascular function, may be defined simply...
as the volume of blood pumped by the heart per unit time. Cardiac output is influenced primarily by four factors: heart rate, myocardial contractility, preload, and afterload (25). Contractility may be defined as the muscle’s intrinsic capacity to do work, independent of the loading conditions. However, the force a muscle generates and its extent of shortening also depend on how much it is stretched before initiation of contraction (preload) and the load against which it contracts (afterload). Current ultrasound techniques do not permit precise delineation of the contribution of each of these parameters to the cardiac output. In addition, ultrasound parameters of cardiac function are all influenced by loading conditions, and many are interdependent. Therefore, we prefer a multi-parameter approach to assessing cardiovascular function, which we believe is less susceptible to the limitations of any single UBM-Doppler measurement. Such an approach can yield much information about cardiovascular physiology, including cardiac output and (systolic) cardiac work, myocardial mechanics, properties of the vascular bed(s), altered blood flow distribution, and characterization of abnormal flow patterns linked with specific genotypes and their morphological phenotypes.

**UBM Indices of Cardiac Function**

Despite some limitations (see below), UBM imaging can provide useful hemodynamic information, not available from any alternative noninvasive imaging approach. The changes in cardiac dimension during contraction may be used to gauge cardiac function (34, 39), while the measurement of vessel size, in conjunction with Doppler flow waveforms, allows calculation of volume flow (33).

Although it is difficult to obtain precise boundaries of each individual ventricle, we have been able to trace the epicardial biventricular areas in embryos staged E10.5–E14.5 in systole and diastole (34, 39). To overcome the poor temporal resolution, we currently record diastolic and systolic ventricular dimensions for at least 10 consecutive cycles and use the maximal and minimal values to estimate the end-diastolic (EDA) and end-systolic (ESA) areas, respectively, and to derive the fractional area shortening \[ \text{FAS} = \frac{\text{EDA} - \text{ESA}}{\text{EDA}} \]. In the unseptated embryonic heart, biventricular fractional area shortening also serves as a surrogate for cardiac ejection fraction. From end-diastolic biventricular area, we can gauge whether there is cardiac dilatation, which should be observed in some, although not all, forms of congestive cardiac failure.

Finally, UBM can detect gross defects, such as pericardial effusions and general ascites (hydrops) of the embryo, which are signs of elevated central venous pressures and diastolic cardiac failure.

**Doppler Indices of Cardiovascular Function**

Doppler waveforms, acquired from the developing embryonic and extra-embryonic circulatory systems, provide a wealth of information pertinent to in utero mouse cardiovascular function (6, 13, 30, 35). In this section, we outline the main parameters currently measured with Doppler, and further discuss the functional significance of a number of these parameters in subsequent sections. Figure 7 provides schematic Doppler waveforms, defining the parameters discussed below.

**Cycle length/heart rate.** Cycle length (CL) is defined as the total time of one complete ejection cycle (in seconds), from the onset of one beat’s flow to the onset of the next beat’s forward flow. Heart rate (HR) is derived simply from CL, as HR = 1/CL (in reciprocal seconds, s⁻¹), or more commonly as HR = 60/CL (in...
beats/min). We have been able to discern clear Doppler signals from mouse embryos as early as the 7-somite (early E8) stage (23), thus providing accurate heart rates from the onset of heart beating through mid-gestation (30).

**Peak velocity.** Peak velocity (PV) is the maximum detected blood velocity (in mm/s).

**Acceleration time/deceleration time.** Acceleration time (AT), also called rise time, is the time from onset of systolic forward flow to peak velocity (in ms). Conversely, deceleration time (DT) is the time from peak velocity to the end of forward flow (in ms).

**Ejection time/non-ejection time.** Ejection time (ET) is the total time of systolic ejection, or forward flow: $ET = AT + DT$ (in ms). Non-ejection time (NET) is the time from the end of ejection to the onset of forward flow of the next beat (in ms). ET may also be normalized to cycle length, as a measure of systolic work.

**Velocity time integral.** Velocity time integral (VTI) is the area under the spectral Doppler flow velocity envelope, when traced along its outer edge (in mm).

**Pulsatility indices.** Pulsatility indices are used, especially in the characterization of umbilical flow dynamics, and in other vessels where the end-diastolic veloc-

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Fig. 5. Extra-embryonic circulations: umbilical/allantoic (top) and yolk sac/vitelline (bottom) blood flow in an E12.5 mouse embryo. The slanted white lines (near the center of the UBM images) outline the approximate sample volume interrogated by pulsed-wave Doppler. Vessels of the umbilical/allantoic and yolk sac/vitelline circulations can be interrogated separately by UBM image-guided Doppler. Because the respective arteries and veins flow in opposite directions, their Doppler waveforms are distinct and appear on either side of the velocity baseline. The Doppler waveforms show that, as expected at this stage, the umbilical circulation displays greater velocities due to greater blood flow volume. Notably, diastolic arterial velocities in both beds drop to zero, a finding that indicates relatively high “downstream” vascular impedance. However, as gestation advanced, the progressive decrease in placental vascular impedance was reflected in changes in not only the umbilical artery, but also the umbilical vein (30). Scale bar (bottom right of UBM images) = 1 mm. Al, allantoic vessel; Am, amniotic membrane; H, heart; P, placenta; L, an embryonic limb; U, uterine wall; V, vitelline vessel; Y, yolk sac.
ity may not be zero (i.e., may not return to baseline). In this case, the ratio of peak (systolic, S) to trough (diastolic, D) velocities, S/D, or the Pourcelot resistive index, (S

/D)/S, is often used to quantify arterial waveform pulsatility and indirectly provides information on “downstream” vascular impedance. Pulsatility indices in umbilical vein (30) may also help characterize “upstream” placental impedance; in principle, the same concept may be applied to other vascular beds.

Isovolumic contraction time/axisovolumic relaxation time. Isovolumic contraction time (ICT) is the interval between mitral valve closure and aortic valve opening (in ms), whereas isovolumic relaxation time (IRT) is the time between aortic valve closure and mitral valve opening (in ms).

E/A ratio. Ventricular inflow waveforms exhibit a characteristic biphase shape, with the E wave (representing ventricular diastole or “passive filling”) preceding the A wave (representing atrial systole, a late diastolic event, or “active filling”). The ratio of E/A peak velocities is often used as an index of diastolic function.

**TOWARD A SYSTEMATIC APPROACH TO ASSESS EMBRYONIC CARDIOVASCULAR FUNCTION**

Parameters of cardiovascular function may be categorized as measures of blood flow, cardiac systolic and diastolic function, and vascular impedance, although within each of these categories, parameters may overlap with other parameters in other categories. In the following sections, we outline a number of functional measurements that may be made with UBM-Doppler.

**Blood Flow**

Theoretically, flow within a vessel can be calculated if one knows the cross-sectional area of the vessel and the flow velocities within the tube. In human (6, 35) and adult mouse studies (16, 49), certain assumptions are made about blood flow physics and flow characteristics. However, with far lower blood flow velocities in a much smaller vasculature, the embryonic mouse may not allow such assumptions. In a first-order approximation model incorporating UBM-Doppler data, we...
recently concluded that the spatial velocity profile in the mouse embryonic dorsal aorta is likely parabolic, not flat, with laminar flow. Using this model, we estimated that the E13.5–E14.5 mouse embryo generates a dorsal aortic stroke volume of ~0.50 mm³ (33). Furthermore, spectral Doppler analysis, such as that found in UBM-Doppler, appears preferable to analysis using mean phasic Doppler, for calculation of volumetric flow, given the assumptions needed for the latter technique (33). Advances in our understanding of the physics of blood flow in the developing embryo will refine our ability to characterize volumetric blood flow in different vascular beds during cardiac development.

Cardiac Systolic Function: Work vs. Contractility

No ideal index of intrinsic cardiac contractility exists. Investigators employing echocardiography, including UBM-Doppler, have typically used indices of systolic cardiac work as useful surrogates. Doppler assessment of blood flow provides a reasonable assessment of overall ventricular function (16). Ejection time and peak flow velocity have been shown to reflect systolic work and parallel changes in contractility when used serially in the same animal. Changes in flow acceleration (∆velocity/∆time) also correlate with changes in contractility (16). As mentioned above, all such indices are influenced both by loading conditions and by heart rate (16). Acceleration time has been touted as an index of cardiac contractility (46, 47) independent of loading conditions (46). At present, however, it is not known how to normalize acceleration time for changes in heart rate, which increases significantly as gestation progresses (23, 30).

Cardiac systolic work and output increase geometrically between E9.5 and E14.5, by all UBM-guided Doppler indices studied, including heart rate, peak aortic flow velocity, velocity-time integral, and ejection time as a proportion of the cycle length. Maturation of cardiac contractility is less clear, however (30). In another study utilizing a conventional clinical ultrasound system, isovolumic contraction time decreased from mid to late gestation, a finding attributed to increasing ventricular pressures (13). However, isovolumic contraction time is clearly lengthened by increasing afterload, is also dependent on preload (since strength of contraction depends on the preload), and is influenced by the heart rate.

Cardiac Diastolic Function

Diastole is a complex phenomenon that incorporates active ventricular relaxation, passive properties of myocardium, and contributions of atrial systole (11). The most commonly used index of diastolic function is the ratio of the E and A waves across the ativoventricular inflow. Although neither is completely independent of the other, certain abnormal flow patterns typically emerge as “diastolic dysfunction” (i.e., meaning worsening ventricular compliance, or increasing ventricular stiffness) progresses (for review, see Ref. 35). In animal work, a reversed E/A ratio or a monophasic A wave has been taken to indicate a stiff ventricle. Indeed, the progression of patterns in the maturing mouse embryo has been interpreted as support for an increasingly compliant (less stiff) ventricle (13). UBM-Doppler studies of embryonic diastolic function are only recently being undertaken, and preliminary results in our laboratory and others (51) indicate that
biphasic inflow patterns exist from close to the earliest stages of heart development (Fig. 3).

Isovolumic relaxation time is sensitive to the rate of ventricular relaxation but because it is also influenced by aortic diastolic pressures and atrial pressures, is load dependent (16). It should be remembered that cardiac valves do not develop until approximately E13.5 in the mouse embryo. Prior to this stage, regurgitation of blood flow is prevented by the dynamic apposition of the endocardial cushions. We question whether changes in the timing of such cushion apposition may occur independently of changes in functional myocardial maturation, potentially confounding the utility of Doppler diastolic indices.

Peripheral Vascular Impedance and Blood Flow Distribution

Using UBM-Doppler, it should be possible to derive volumetric flows and distribution of blood flow/cardiac output to various intra- and extra-embryonic vascular beds, which may change during normal development (18) and be altered in mouse mutant models. As ejection ends, Doppler parameters will reflect the “downstream” impedance, that is, the impedance of the peripheral vascular bed distal to the point of Doppler interrogation. Thus, for example, the deceleration and non-ejection times of the aortic waveform, and the ratio of the peak-trough umbilical arterial velocities are influenced by the capacitance of their respective “downstream” vascular beds. Venous flow (e.g., umbilical venous) may also provide some insights into “upstream” vascular bed impedance (30). In principle, these techniques should enable us to approximate the relative vascular impedances of the intra-embryonic bed, the placenta, and the vitelline circulation/yolk sac bed (Fig. 5).

Data from the normal mid-gestation mouse embryo strongly suggested, as expected, a progressively higher capacitance and lower impedance of the placental bed (30). Notably, in a study of mouse embryonic umbilical blood flow using invasive techniques, umbilical arterial flow was abolished when the ventricles were artificially paced (27). This finding suggests the intriguing possibility that, in addition to the embryonic circulation’s dependence on an optimum heart rate for efficient cardiac output, the distribution of the cardiac output is exquisitely sensitive to such changes in heart rate and cardiac output.

PUTTING IT ALL TOGETHER: ESTABLISHMENT OF EARLY CIRCULATION

When cardiac function commences and true circulation is established are fundamental questions in development. UBM-Doppler has allowed investigators to record the earliest cardiac contractile and circulatory events in the in utero mouse embryo. Srinivasan et al. (39) and Zhou et al. (51) have demonstrated flow in E8.5 embryos, at approximately the heart tube stage, although these were not staged precisely. More recent data from our laboratory have demonstrated rhythmic cardiac contractions as early as the 5-somite (straight heart tube) stage, with intra-embryonic blood flow evident by 7–8 somites (23). Notably, the onset of cardiac activity correlated precisely with entry of red blood cells into the embryo proper. All indices of cardiac systolic work increased dramatically through E10.5 and aligned nicely with previous UBM-Doppler data obtained from mid-gestation mouse embryos (30). These results, the first obtained in utero, indicate that establishment and early development of the circulation are precisely coordinated, bringing together cardiac activity, an intact vascular circuit, and oxygen-carrying red cells to support the growing embryo (23).

ABNORMAL BLOOD FLOW PATTERNS AND PHYSIOLOGY

The ability to correlate a detailed cardiovascular physiological phenotype with a given genotype is an exciting development in functional genomics. To date, however, only scant data have been reported in models of abnormal mouse cardiovascular development. Several investigators (13, 21, 28, 40) have utilized the Doppler capabilities found in conventional human echocardiography systems. Gui and colleagues (13) demonstrated presumed atrioventricular regurgitation in mid-gestation trisomy 16 mouse embryos on Doppler waveform analysis. Huang et al. (21) and Sullivan et al. (40) detected increased blood flow velocities, presumably in the pulmonary outflow, in mouse embryos exhibiting abnormal gap junctional communication, including the connexin 43-overexpressing transgenic (CMV43) and connexin 43 knockout mouse. Changes in other Doppler functional parameters were also observed. It should be noted, however, that in these experiments, precise localization of abnormal Doppler signals to the presumed abnormal region was not possible, given the large Doppler sample volume and poor spatial resolution of the ultrasound system. Maki et al. (28) demonstrated abnormal pulsatility with semilunar valve regurgitation in late gestation (E18.5) lox−/− mouse fetuses, which have structural alterations in arterial walls leading to aortic aneurysms. Interestingly, lox−/− fetuses demonstrated normal-appearing cardiac structures and dimensions, despite Doppler evidence of reduced cardiac output and right heart failure. The ability to localize abnormal flows in this study was possible only because of the size and gestational age of the mouse fetuses, which were well beyond cardiac developmental stages. Disturbances in cardiac rhythm may also be detected with Doppler. Huang and Linask (22) found that tribromoethanol anesthesia could induce premature atrial contractions in mid to late gestation mouse embryos. Premature atrial contractions also seemed to be more prevalent in CMV43 mouse embryos (21). Despite limitations of spatial resolution and localization of Doppler abnormalities, these studies demonstrate the potential of even conventional clinical ultrasound systems, in studies of the developing mouse.
In our laboratory, we have previously demonstrated the ability of UBM to detect depressed ventricular function and pericardial effusions, indicative of heart failure, in the embryonic-lethal VCAM-1 homozygous null mutant mouse (39). In other experiments, we have also noted arrhythmias, including bigeminal rhythms and progressive bradycardia in dying embryos (CKL Phoon, O Aristizábal, RP Ji, and DH Turnbull, unpublished observations). Experiments are currently underway on other models of abnormal cardiovascular development (31, 34). Detection of abnormal cardiovascular physiology is but a first step; elucidation of the mechanisms underlying embryonic disease and death will require a comprehensive and multi-parameter UBM-Doppler approach (32, 34).

ADDITIONAL COMPLICATING FACTORS

Despite the successes of UBM-Doppler in providing in vivo data relevant to cardiovascular function in developing mouse embryos, it must be realized that there are several potentially complicating factors involved in the acquisition of these data.

The low frame rate of currently available, mechanically scanned UBM systems (8–10 images/s), in the context of normal embryonic heart rates of 3–5 beats/s, limits its temporal resolution. This problem will only be overcome by the future development of higher frame rate UBM systems, via the introduction of either faster mechanical scanners or high-frequency phased-array transducers (see Ref. 45, also http://www.visualsonics.com). Furthermore, the spatial resolution of UBM (e.g., 30 μm axial and 90 μm lateral resolution at 40 MHz), while excellent compared with clinical ultrasound systems, is still marginal for mouse embryo work, where, for example, the dorsal aorta diameter is ~300 μm. Therefore, the ability to gauge precisely cardiac dimensions throughout the cardiac cycle is suboptimal. Moreover, at the high UBM-Doppler frequencies used (40–50 MHz), blood is echo-dense, and the endocardial lumen (and hence myocardial wall thickness) cannot be visualized in the early to mid-gestation mouse embryo (39, 51). Interestingly, this problem appears to resolve somewhat in late gestation, so that it may be possible to characterize intracardiac anomalies later in gestation (51).

Anesthesia, although necessary to acquire reproducible data, may affect physiological measurements. The ideal anesthetic agent and regimen for mouse cardiovascular work have not been identified. However, recent data in early to mid-gestation mouse embryos showed that embryonic and maternal heart rates did not change significantly between the start and the end of experiments. These data indicate that heart rates, which are exquisitely sensitive to cardiovascular perturbations, were not significantly altered by differences in anesthesia level, which are expected to change significantly over the 1-h examination period, since the mouse is typically deep under anesthesia at the start, and starting to regain consciousness toward the end of the examination (23). Further research will be required to determine the effects of various anesthetic regimens on a number of UBM-Doppler parameters in the developing embryo.

Several experiments in our laboratory currently employ a “semi-invasive” approach, in which the intact uterus is surgically exteriorized into a bath of physiological saline (at 37 ± 1°C) for UBM-Doppler, maintaining all maternal, embryonic, and extra-embryonic circulatory systems intact (23, 31, 33, 34). This approach has been adopted to enable easier acquisition of high-quality UBM-Doppler data from entire litters of embryos, both at very early development stages (23) and in the characterization of mutant phenotypes (31, 34), although it has the obvious limitation of being less amenable to longitudinal studies. Comparison of data acquired noninvasively and semi-invasively shows that, although the heart rates are slightly lower in the exteriorized embryos, other Doppler parameters are not significantly different under the two conditions (23). Moreover, observed embryonic heart rates (both semi-invasive and noninvasive, and under anesthesia) are significantly higher than others reported in the literature (23, 30), leading us to believe that these data are closer to the physiological situation than those in previous reports using more invasive measurement techniques.

It should be noted that analytical techniques used in human (6, 35) and adult mouse (16) studies are not necessarily applicable to the embryonic mouse. Continual refinements in UBM-Doppler data analysis (30, 33) as well as technology (1, 10, 51) will be required for UBM-Doppler to fully realize its potential to shed insights into developmental cardiovascular physiology.

OTHER VERTEBRATE MODELS OF CARDIAC DEVELOPMENT

Although the mouse remains the preferred model of mammalian development, other animal models have also contributed to our understanding of cardiovascular development and are potential candidates for UBM-Doppler imaging. For example, the chick embryo has been a favorite vertebrate model for developmental physiologists because of its ease of access and ability to be surgically manipulated and instrumented (see Ref. 3). However, to our knowledge, the chick embryo has yet to be studied by UBM-Doppler or other non-invasive ultrasound techniques. The exposed chick embryo, however, is amenable to optical imaging techniques that afford superb spatial and temporal resolution (50), so that the potential role of UBM-Doppler is not entirely clear.

With large-scale genetics screens available, the zebrafish has become a popular vertebrate model with which to identify and study genes involved in cardiac development (12, 48). Evolutionary conservation means that much of zebrafish cardiac development is relevant to mammalian cardiac development, even though the fish heart is two-chambered. Notably, the early larval fish can survive through diffusion of oxygen alone, without the need for a
functional cardiovascular system; thus mutations that would lead to early embryonic lethality in the mouse may still be characterized in early stage zebrafish. Also, morphogenesis and function have been easier to study than in the mouse because of the transparency of the young fish (12, 48). Cardiovascular function in both the developing and adult zebrafish has been studied using high-speed videomicroscopy and servo-null micropressure systems (12, 19, 20, 48). Recently, Hove et al. (17), using high-speed fluorescent confocal in vivo imaging and surgical manipulation, demonstrated the important role of shear forces in normal cardiac development. To date, the use of Doppler ultrasound has been limited to the study of adult zebrafish (15).

With the advantages of the zebrafish model in mind, we have begun to explore the feasibility of characterizing zebrafish cardiovascular hemodynamics using UBM-Doppler, especially in the developing heart. In preliminary studies, we have been able to obtain Doppler signals from the aorta at various stages from ~48 h postfertilization through adulthood (Fig. 8; CKL Phoon, RP Ji, O Aristizábal, D Yelon, and DH Turnbull, unpublished observations), and at temperatures of 25–30°C. One major difference between fish and mice is that fish are poikilothermal (cold-blooded), so that there is no single “physiological” temperature (15). One will therefore need to exercise caution in comparing cardiovascular data from different studies, since even small differences in temperature exert substantial influences on cardiac function (15, 32). In our hands, the consistent acquisition of Doppler signals in zebrafish has been challenging; we believe that a small aorta, lower blood flow velocities, and the high reflectivity of fish skin/scales contribute to a low signal-to-noise ratio and suboptimal Doppler signals. Nevertheless, with continued refinement of techniques, the application of UBM-Doppler to the study of zebrafish mutations is likely to yield further insight into functional genomics, complementing information derived from other techniques (17, 19, 20) and that from the mouse.

**SUMMARY AND FUTURE DIRECTIONS**

UBM-Doppler has become an important technique in the analysis and characterization of developmental cardiovascular physiology. Challenges ahead in UBM-Doppler imaging include the development of higher-resolution (both spatial and temporal) technology, improved Doppler signal analysis, and advances in the understanding of microcirculatory flow physics. Investigators are now beginning to move from largely descriptive studies of cardiovascular physiology to addressing important developmental questions, in both normal and abnormal models. Insights into mechanisms of development, altered physiology, and embryonic compensatory responses will ultimately necessitate the use of a variety of imaging modalities, in conjunction with invasive physiological assessment and cellular biology techniques. Nevertheless, the distinct advantages of UBM-Doppler as a non-invasive imaging technology able to characterize mammalian embryonic hemodynamics assure it a central role in the field of developmental cardiovascular physiology.

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**DISCLOSURES**

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**Fig. 8.** UBM-Doppler imaging of zebrafish. As can be seen, the zebrafish is much smaller in its larval stages than the developing mouse embryo. Here, we show arterial flow signals from the zebrafish aorta at 48 h postfertilization (48 hpf), whose velocities are far lower than are typical in the mouse embryo. The slanted white lines (near center of the UBM image) outline the approximate sample volume interrogated by pulsed-wave Doppler. Scale bar (bottom right of UBM image) = 1 mm. Gelatin agar (G) is used to position and hold the zebrafish in place during imaging; H, head; P, petri dish bottom; T, tail.
REFERENCES


