ABNORMALITIES IN GROWTH and development of the cardiovascular system are among the most common congenital birth defects (28, 29) and show a large degree of variability, which indicates that cardiovascular development relies on a complex set of signals. With the use of model systems such as mice, great strides are being made in elucidating the molecular signaling pathways involved in early heart and blood vessel formation (for a review, see Ref. 6). Through both single-gene targeting approaches and large-scale screens (for a review, see Ref. 21), a growing number of mutants are being identified with defects in hematopoiesis (33, 36, 42), endothelial cell formation and organization (2, 13, 37), and cardiogenesis (6, 25, 41).

In addition to genetic regulation, mechanical signals imparted through frictional forces created by blood flow are also essential for normal development. If flow is interrupted during embryonic development, heart and vascular defects arise (1, 3, 16, 17, 19, 28, 41). In vitro studies have revealed that the shear stress caused by fluid flow can induce changes in the morphology and activity of cultured endothelial cells and cause cell alignment, proliferation, and activation of gene expression and enzyme function (14, 22, 27, 35, 38). Interestingly, several gene products whose expression and activation are influenced by flow in cultured endothelial cells are also necessary for development (22, 27, 32, 35). All of these data suggest that shear stress may act as a key regulator of development, but very few studies have related shear stress levels to specific cellular events in the embryo.

A large gap exists between the precisely controlled flow and quantitative evaluations performed in vitro and the complex dynamic interactions that occur during early circulation in vivo. This is largely because methods for quantitative hemodynamic analysis of newly formed vessels in vivo have not been established. In this study, we show that quantitative flow measurements can be made in early mouse embryos at a time when the cardiovascular system is first forming. With the use of confocal laser-scanning microscopy (CLSM) to image mouse embryos (days 8.5–10.5) that express green fluorescent protein (GFP) in primitive erythroblasts (11), velocity measurements were made in vessels ranging from the diameter of a single blood cell (10 μm) up to several hundred micrometers. Close inspection of velocity profiles revealed that both laminar and disturbed flows are present in the early yolk sac. Therefore, cells are exposed to both laminar and oscillatory shear stress during development. In support of the hypothesis that mechanosensory signals play a role in development, laminar shear stress (LSS) levels were consistent with the shear stress levels known to affect the morphology and molecular signaling of endothelial cells in vitro. Thus it is possible to use quantitative methods to study the remodeling of embryonic vasculature. Moreover, changes in the acceleration and deceleration of flows were observed as the hearts matured, which shows that velocity measurements can be used to study heart development. The model system described here in combination with mutant analysis provides a powerful approach to studying cardiovascular development and the role of mechanosensory signals.

METHODS

Dissection. The investigation conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Homozygous e-globin:GFP male breeder mice (11) were mated with CD-1 females overnight. The presence of a vaginal plug was taken as 0.5 days postcoitum (dpc). Embryos were collected on the morning of the eight, ninth, or tenth day in a Plexiglas hood heated to 37°C using a chicken incubator heater (model 115-20, Lyon Electric). Dissecting medium consisted of 90% DMEM/F-12 (catalog no. 11330032, GibCO), 8% heat-inactivated FBS (catalog no. 16140063, GibCO), 1% of 1 M HEPES buffer solution (catalog no. 9319, Irvine Scientific), and 1% Pen-Strep solution (catalog no. 9366, Irvine Scientific). The medium was warmed to 37°C before dissection. Females were

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Fig. 1. Line-scanning image to measure blood flow. Line scans were performed perpendicular to the direction of blood flow on transgenic embryos that express green fluorescent protein (GFP) in their primitive erythrocyte cells (11). White line represents the location of the scans within an x-y image of the yolk sac (A). Green fluorescence represents red blood cells, and the red color is CellTracker Orange staining, which was used to visualize the vessel walls. Line scanning at this location yields a length (L) vs. time (t) image (B). Several measurements were made on these L vs. t images including the L and r locations of the beginning of the streak, the L and r dimensions of the streak, and the diameter of the streak (C).

To determine $V_x$, which is the component of the velocity perpendicular to the laser line, the distance traveled is divided by the time taken to pass over the laser line

$$V_x = \frac{D \times S_{\text{pixel}}}{(\Delta r - 1) \times V_{\text{scan}}}.$$

If there is also a component of the velocity parallel to the laser line, it can similarly be calculated using

$$V_y = \frac{\Delta L \times S_{\text{pixel}}}{(\Delta r - 1) \times V_{\text{scan}}}.$$

where $\Delta L$ is the measured displacement in the L direction.

Using these equations, the overall velocity is given by

$$V = \frac{S_{\text{pixel}} \sqrt{\Delta L^2 + D^2}}{(\Delta r - 1) \times V_{\text{scan}}}.$$

Clumps of primitive erythroblasts could not, in general, be analyzed using this technique. It was necessary to disregard a small subset of erythroblasts whose edges could not be clearly distinguished.

Line-scan images where the erythroblasts passed over the laser line in fewer than three scans were discarded. Under these circumstances,

primitive erythroblasts are spherical (Fig. 2). Regardless which z-plane of the cell is imaged, the distance traveled is equivalent to the cell diameter $D$ measured in pixels. Therefore, the distance traveled is given by

$$\text{Distance} = D \times S_{\text{pixel}},$$

where $S_{\text{pixel}}$ is the pixel size (in μm/pixel).
diameter measurements may be inaccurate, because it is impossible to know whether the laser had imaged the erythroblast close to the center of the cell. The top line-scanning rate of the Zeiss LSM PASCAL microscope is 384 μs/line for a line that is 512 pixels long. For a red blood cell with a diameter of 10 μm, the theoretical maximum velocity that can be measured is 13,021 μm/s if the vessel diameter is <20 μm wide (using a ×20 magnification lens) or 10,400 μm/s if the vessel is ≤460 μm in diameter.

Validation of method. To validate these measurements, a mass balance on merging yolk sac vessels was calculated. Because inflow must equal outflow, the sum of the volumetric flow rate of the two input vessels at a bifurcation should equal the volumetric flow rate of the larger vessels into which the two smaller vessels merge (as long as differences in density are negligible).

Assuming a parabolic profile, the maximum velocity is twice the average velocity, and so the volumetric flow rates (Q) can be calculated from

\[ Q = \frac{V_{\text{max}}}{2} \pi r^2 = V_{\text{avg}} \pi r^2 \]

The pulsatile nature of the flow can be ignored for this calculation, because flow within the embryonic vasculature conforms to the Stoke’s flow regime (Reynold’s number ≪ 1), and the Womersley parameter is known to be ≪ 1 (31).

Because line scans were performed sequentially, a simultaneous comparison of flow rates could not be made. Therefore, the volumetric flow rate was calculated using two different methods to verify that the calculations were unbiased and the results from each method were comparable with very little error. In the first method, the average of the top 2% of all measured velocities for a vessel was taken as \( V_{\text{max}} \). In the second method, the mean velocity of all the flow rates was taken as \( V_{\text{avg}} \). The error associated with the mass balance (5–7%; data not shown) was minimal, which indicates a high level of accuracy in the measurements. Errors in individual velocity measurements were minimized by averaging multiple determinations.

To analyze the components that introduced the most error, a sensitivity analysis was performed. Errors in scan rate and pixel size are related to the microscope software and are expected to be minimal. The most sensitive variable to measurement error was the \( \tau \)-dimension of the streak. At the fastest flow rates that could be measured, streaks often had <5 line scans; this could easily introduce significant error especially if the length was underestimated. The strength of this method, however, is that by measuring every single red blood cell, so many measurements are made that errors are minimized through data regression.

Calculation of hematocrit. A scan through the center of the vessel was used to represent the flow present in the whole vessel because nondisturbed laminar flow is parabolic (see Fig. 4). Our calculations assume that the vessel is spherically cylindrical, because the difference in dimension on each axis is on the scale of one or two red blood cells. The center of a vessel along the \( z \)-axis was determined by changing the focal plane to reveal the widest cross section. The hematocrit (Hct) was calculated by first cropping the images to include only regions of blood flow as assessed by the location of the vessel walls. The image was then converted to gray scale, and a threshold was applied to the image such that pixels were either pure white or pure black. The intensity at which the threshold was applied was chosen by increasing the zoom on the image to visualize the individual pixels and finding the level at which the size of the red blood cell streaks between the black-and-white and gray-scale images were in agreement. The percentage of white pixels within the image was used as the Hct measurement.

Calculation of LSS. The velocity data during a pulse was plotted vs. \( \tau \)–position within the line-scan image to yield the velocity profile within the blood vessel (as in Fig. 4). The location of the pulse was assessed visually, and only the velocity data during the peak flow were used. The data were regressed using the parabolic profile

\[ V = ar^2 + b \]

where \( V \) is the velocity, \( r \) is the radial position of the measurement, and \( a \) and \( b \) are the regression results.

The vessel radius, \( R \), is found from the \( x \)-intercept

\[ R = \sqrt{-\frac{a}{b}} \]

This value was compared with the measured diameter based on CellTracker Orange staining. If there was a large difference between actual and calculated diameters, data from that pulse were rejected. This occurred in <10% of the scans and was generally caused by blood flow in which all the red blood cells were located near the center of the vessel. The shear rate (\( \dot{\gamma} \)) was calculated based on the slope of the regressed line at the vessel wall

\[ \dot{\gamma} = -\frac{dV}{dr} \bigg|_{r=R} = -2aR \]

The Hct was calculated by counting the number of fluorescent cells within the boundaries of the vessel wall.

The apparent viscosity for embryonic mouse blood was approximated using data from adult human blood (4), because no relevant work has been done on embryonic mouse blood. The apparent viscosity of human blood is very likely to be similar to embryonic mouse blood despite the fact that red blood cells in mouse embryos are nucleated. Studies have shown that at low shear rate and low Hct value, viscosity is comparable between nucleated avian blood and enucleated human blood (40). The shear rate data and the Hct data for each pulse were inserted into a sixth-order logarithmic regression for calculating blood viscosity at low shear rates (4).

The shear stress (\( \tau \)) is calculated by multiplying the shear rate by the apparent viscosity (\( \mu_{\text{app}} \))

\[ \tau = \mu_{\text{app}} \dot{\gamma} \]

RESULTS

Blood velocity measurement in embryonic yolk sac microvasculature. We focused our studies on blood flow in the developing yolk sac of early mouse embryos. In mammals, the first site of red blood cell production is in the yolk sac, an extraembryonic multilayered membrane that surrounds the embryo proper. Both blood and endothelial cells differentiate in the yolk sac before the development of a functional heart (beginning at 7.5 dpc in mice), but vasculogenesis and vessel remodeling occur after the heartbeat starts and blood flow begins.

The yolk sac is an excellent model for studying early hemodynamics, because it is a simple system that consists of only a few different cell types compared with the adult; yet in a few short days, vascular development can be observed from the initial differentiation of endothelial cells through the formation of remodeled vessels. In addition, by studying fluid dynamics in yolk sacs, movement artifacts produced by the heartbeat are minimized. To aid in visualizing blood flow in yolk sacs, whole mouse embryos that expressed GFP in primitive erythroblasts (11) were grown in culture on the stage of a confocal laser-scanning microscope (20).

To examine flow regimes and quantify the level of shear stress, the velocities of erythroblasts within the vessel and the Hct must be determined. To accomplish this, we adapted a technique originally developed for measuring blood flow in
adult microcirculation in which a confocal laser-scanning microscope is used to scan a single line parallel with the flow in a vessel to measure flow velocity (10). We altered the technique by scanning perpendicular rather than parallel to the direction of flow to measure vessel size. Hct, and the flow profile through the vessel, all of which are necessary to calculate shear stress.

In traditional CLSM, a laser that excites a given fluorochrome in a cell is scanned across a field of view while a detector simultaneously collects the emission signal, pixel by pixel, to produce a two-dimensional image (see Fig. 1B). For instance, for a single image that is 512 × 512 pixels, the laser scans across the field of view, collects 512 pixels worth of data in a straight line, and then moves to the next line to acquire the next row of pixels. Therefore, the time to acquire the whole image depends on the acquisition time for each line, the number of lines, and the time it takes for the laser to turn around to scan the next line. Scanning the whole field of view is often too slow for capturing very rapid dynamic events, but line scanning (repeatedly scanning a single line) can be used as an alternative if enough image data are gathered from a single row of pixels.

For the method described here, the laser was used to repeatedly scan along a single line positioned perpendicular to vessels of various sizes in mouse yolk sacs (see Fig. 1A, white line). The scan line acts like a photo-finish camera at a race, imaging a single row of pixels and collecting partial images of blood cells as they pass that point in the vessel. Slower moving blood cells are imaged more often than rapidly moving cells as they cross the scan line. Velocity can be measured by determining how many times the same cell was imaged and thus how long it took for the cell to cross through the line scan. To determine the velocity of a cell, line scans are reconstructed as two-dimensional images (L vs. t; see Fig. 1B). The length of the streak that is produced by the image of the cell can be directly related to velocity given that the line-scanning speed, pixel size, any movement perpendicular to the vessel, and the cell diameter are known (see Fig. 1C; see also METHODS). This calculation is simplified by the fact that early embryonic erythroblasts are spherical throughout all embryonic stages imaged (see Fig. 2) and do not appear to deform significantly during flow (data not shown). Therefore, the optical plane of the cell being imaged is inconsequential, because the two-dimensional image of the cell will always be circular, and the distance traveled can be related to the diameter.

With the use of this method, the minimum velocity that can be measured is determined by the number of scans, whereas the maximum limit is determined by the scan speed of the instrument (see METHODS). Routinely, 3,000 lines are scanned, yielding a theoretical lower limit of 3.7 μm/s, whereas the fastest velocity that can be measured by our present system is 13,000 μm/s. In yolk sacs, measured flow velocity ranged between 18.5 and 6,000 μm/s. Because measurements of the fastest velocities in yolk sacs were well under the theoretical limit, we made velocity measurements in the dorsal aorta at 8.5 dpc, where measurements showed that flow traveled up to 9,400 μm/s. To determine the error rate for these measurements, a mass-balance analysis was performed (see METHODS). We observed <7% error, which indicates that this method provides valid measurements of flow velocity.

Line scanning was used to assess the velocity of the blood in yolk sac vessels in embryos at 8.5, 9.5, and 10.5 dpc (Fig. 3). We observed a range of velocities in the embryos at the three stages examined. At the earliest stage examined, just after circulation began, the channels in the vascular plexus were of similar size, yet flow velocities were found to be quite variable. At 9.5 and 10.5 dpc, vessels varied in size from ~50 to 200 μm. At these stages, both velocities and vessel sizes are highly variable, but blood flow appears to travel at similar velocities in vessels of the same size. It is unknown whether velocity at 8.5 dpc is a predictor of vessel size at later stages (see DISCUSSION).

![Fig. 3. Comparison of velocities at different embryonic stages. Blood flow velocity vs. vessel diameter was plotted for 8.5-dpc (A), 9.5-dpc (B), and 10.5-dpc (C) embryos. $V_{\text{max}}$ represents the maximum velocity during the pulses, which was calculated by regressing the velocity profile according to a parabolic profile.](http://ajpheart.physiology.org/)
Determining blood flow profiles in developing yolk sacs. Line scanning perpendicular to the vessel was used to obtain flow profiles from vessels of yolk sacs. Flow profiles are used to show directionality with respect to vessel structure. Although it is generally assumed that early embryonic blood flow is laminar (31), this has not been shown directly. Laminar flow is characterized by steady movement with a straight trajectory without mixing. Flow velocity has a parabolic profile with respect to vessel diameter such that cells in the middle of a vessel travel faster than cells slowed by friction near the vessel walls. In addition to laminar flow, blood flow can exhibit more complex profiles. Yolk sac vessels are too small and flow is too slow to produce turbulence, but areas of flow separation and vessel branching can produce eddies or other disturbed flows. Disturbed flows can be characterized by cells moving in irregular or circular motions within the vessel. Both laminar and disturbed flows were detected within yolk sacs. Figure 4A shows the L vs. t plots of steady flow along a single axis: cells move predominately along the axis of the vessel parallel to the wall with limited movement along the perpendicular axis. Velocity profiles of steady-flow regions are parabolic at peak (systole) and nonpeak (diastole) velocities (Fig. 4B), which confirms that flow is indeed laminar in these regions. Figure 4C shows the L vs. t plot of a region where an eddy is present. A disturbed pattern of flow is observed where the relative angle of the line-scan streaks change with respect to one another. Velocities plotted along the diameter of the vessel do not fit a parabolic profile (Fig. 4D) and therefore are nonlaminar.

Hct measurements in embryonic circulation. To calculate shear stress, Hct as well as flow velocity and flow profile are needed. Hct was measured directly in individual yolk sac vessels from 8.5 to 10.5 dpc. Figure 5A shows Hct values at different stages of development. Hct was observed to increase slightly between 8.5 and 10.5 dpc from ~15 to 20% of total intravascular volume. Furthermore, there is a linear relationship between vessel diameter and Hct in individual embryos that is statistically significant (average P = 0.074) and is independent of developmental stage (Fig. 5B). This relation-
ship between vessel size and Hct is called the Fahraeus-Lindqvist effect (12) and has never been reported in an embryonic system.

**LSS in embryonic flow.** The LSS levels were calculated in different-sized vessels at different times during development (Fig. 6) using a correlation to approximate blood viscosity (4) based on shear rate and Hct (see METHODS). LSS values ranged from 0.40 to 5.16 dyn/cm², which are consistent with shear stress levels known to modulate the expression and subcellular localization of proteins important in normal vascular development such as Flk-1 and other growth-factor receptors. At 8.5 dpc, the levels of shear stress were not related to vessel size, whereas at both 9.5 and 10.5 dpc, shear stress values were higher in larger vessels. In addition, the magnitude of shear stress increased from 9.5 to 10.5 dpc, which is a time when vessels undergo remodeling.

**Change in flow with heart development.** Between 8.5 and 10.5 dpc, the heart is transformed from a linear tube to a looping heart that consists of multiple chambers and developing valve primordia (endocardial cushions). To determine how the periodicity of flow changes during heart development, blood velocity values were plotted with respect to time (Fig. 7). Yolk sac vessels were compared that had approximately equal maximum velocities. For the earliest embryos examined (8.5 dpc), when the future heart was still a linear tube, velocity increased and decreased gradually with an irregular frequency (Fig. 7A). For 9.5-dpc embryos, in which the endocardial cushions were more pronounced, velocity increased steeply and abruptly and decreased slowly thereafter (Fig. 7B). Finally, for 10.5-dpc embryos, velocity increased steeply and rapidly and then decreased sharply (Fig. 7C). These changes in acceleration and deceleration of the flow cycle likely reflect the improvement in heart function from 8.5 to 10.5 dpc.

**DISCUSSION**

Here, we present novel measurements of fluid dynamics in the early mammalian cardiovascular system. Using confocal line scanning, we can accurately measure flow velocities, flow profiles, and Hct levels in embryonic microvasculature. We have characterized changes in blood flow through yolk sacs of early mouse embryos and have calculated levels of shear stress induced by blood flow.

**Fig. 6.** Shear stress values. Shear stress was calculated in vessels of different sizes at three stages of development. Shear stress in older embryos [9.5- and 10.5-dpc embryos (E9.5 and E10.5, respectively)] increased with increasing vessel diameter. Shear stress values obtained before the capillary plexus had remodeled [8.5-dpc embryo (E8.5)], however, were more scattered and showed no defined pattern.

**Fig. 7.** Blood flow velocity changes with heart development. Values for measured velocity vs. time were plotted for 8.5-dpc (A), 9.5-dpc (B), and 10.5-dpc (C) embryos. Pulse is visible at all ages; however, it becomes more discrete as embryos develop.

**Primitive erythroblast movement during yolk sac development.** In mouse embryos, blood cells continue to differentiate and proliferate as circulation begins. Hct measurements in the early embryo increased steadily as the embryos developed. In addition, even at the earliest stage examined, the Hct value was directly dependent on vessel size. In larger vessels, we observed a higher percentage of erythroblasts. This relationship, the Fahraeus-Lindqvist effect (12), has previously been dem-
onstrated in vivo in adult tissues (24) but has never been reported in embryos. Although individual vessels in a single embryo can vary in size up to threefold, the relationship between vessel size and Hct is maintained.

The Hct measurements presented here reflect those cells that were actively moving through the vasculature. Consistent with previous studies (26), we also observed many primary erythrobasts that did not circulate freely (data not shown). In fact, in some cases, primary erythrobasts remained at the site where they formed, and others were carried to new sites and became trapped. It is not known whether the fate of circulating vs. noncirculating blood cells is different. For example, noncirculating blood cells eventually may be eliminated by programmed cell death or they may fail to proliferate.

Flow patterns and shear stress during embryonic development. We measured LSS levels between 0 and 5.5 dyn/cm² in embryos from 8.5 to 10.5 dpc. Similar levels of LSS have been shown to induce notable responses in cultured endothelial cells. In vitro, shear stress levels are associated with endothelial cell alignment and have been shown to induce the expression of numerous transcription factors, cell adhesion molecules, enzymes, and cell signaling molecules including growth factors and growth-factor receptors that are known to play a role in development (for a review, see Ref. 39). For instance, noncirculating blood cells eventually may be eliminated by programmed cell death or they may fail to proliferate.

In addition to laminar flow, we also detected eddies or disturbed flows. From in vitro studies, it is known that laminar and disturbed flows produce different types of force or shear stress that in turn have differential effects on endothelial cells. Oscillatory shear stress (OSS) induced by eddies (5) correlates with increased cell proliferation. In fact, both the magnitude of shear stress and local fluctuations in shear stress may be important mechanosensory signals (5). Cell proliferation in addition to other factors is thought to contribute to the formation of atherosclerotic plaques that often form at branch points in arteries (8). In development, OSS may play a part in vessel growth at the branch point or may signal the proliferation of endothelial cells during vasculogenesis and remodeling. Although it is possible to calculate the magnitude of laminar stress using line scanning, the line-scanning method cannot be used to determine the level of OSS. Continuous particle tracking is needed to measure forces exerted by particles with circular or irregular trajectories. Others have made measurements of OSS and even turbulent flows using high-speed imaging and correlation techniques such as digital particle image velocimetry (17); however, high-speed fluorescence imaging is often hampered by poor sensitivity and may not be feasible for these studies.

Hemodynamics and cardiac development. Recently, there has been increased interest in the relationships between blood flow and heart and vessel development. In zebra fish, it has been shown that blocking fluid flow and thereby reducing shear stress results in heart defects (17). In silent heart mutants, where flow is impaired (19), patterning defects are present in the intersegmental arteries. In mice, the heart does not form properly if flow is stopped through ablation of Ncx1, a Na⁺/Ca²⁺ exchanger (41). Also, in myosin light chain-2a mutant embryos, the atria and endocardial cushions do not develop properly (18). It is noteworthy that these mice also have defects in the yolk sac vasculature, although the myosin light chain-2a gene is only expressed in cells within the heart. These and other mutants provide a unique opportunity to study the relationship between fluid dynamics and vascular development, because flow is disrupted in a way that does not also directly interfere with development of endothelial cells.

In addition to studying shear stress, we can observe subtle defects in valve function and cardiac muscle development by examining the periodicity of flow through the heart, which provides us with greater insights into how flow is disrupted in the presence of cardiac abnormalities. The measurements of flow periodicity shown here are similar to the Doppler waveforms produced by ultrasound (30) but can be obtained using a confocal microscope, which is more readily available in many research labs.

Mechanosensory signals and vascular remodeling. The data we describe here show that it is possible to explore the relationship between mechanosensory and biochemical signals involved in vessel development and patterning at a cellular level. We have shown that mechanical forces that act on endothelial cells can be measured using CLSM. CLSM is also a powerful tool for imaging changes in endothelial cell morphology and expression of signaling molecules, which makes it possible to study how variations in blood flow affect vascular remodeling. Thus we have developed a robust model system for studying how mechanosensory signals function in the development of the mammalian cardiovascular system.
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