Developmental Changes in Ventricular Diastolic Function Correlate With Changes in Ventricular Myoarchitecture in Normal Mouse Embryos

Takahiro Ishiwata, Makoto Nakazawa, William T. Pu, Sergei G. Tevosian, Seigo Izumo

Abstract—Both genetic and epigenetic factors, such as abnormal hemodynamics, affect cardiac morphogenesis and the pathogenesis of congenital heart disease. Diastolic function is an important determinant of cardiac function, and tools for evaluating diastolic function in the embryo would be very valuable for assessment of cardiac performance. Using histological measurements of ventricular myoarchitecture, Doppler assessment of ventricular inflow velocities, and direct measurement of ventricular pressure, we investigated developmental changes of ventricular diastolic function in the mouse embryos from embryonic days 9.5 to 19.5. Regression analysis showed that peak velocity of A wave (an index of passive compliance) correlated with the area of trabecular myocardium in right ventricle (RV) \( r^2 = 0.92, P<0.0001 \) and left ventricle (LV) \( r^2 = 0.93, P<0.0001 \). Peak velocity of E wave (an index of active relaxation) exponentially correlated with the area of compact myocardium in RV \( r^2 = 0.98, P<0.0001 \) and LV \( r^2 = 0.97, P<0.0001 \). We used these techniques to analyze FOG-2 null embryos. FOG-2 null embryos had thin compact myocardium, higher EDP and E/A ratio, smaller \( -dP/dt \), and diminished sucking pressure than wild-type littermates, indicating that decreased ventricular diastolic function might be the primary cause of embryonic lethality. In conclusion, during embryogenesis the development of compact myocardium tightly regulates the development of ventricular distensibility. Our study in normal mice forms the basis for future studies of embryonic cardiac function in genetically manipulated mice with abnormalities of the cardiovascular system.  

Key Words: cardiac development • embryonic circulation • diastolic function • myocardial architecture • gene targeting mouse

Inadequate cardiac performance as a result of congenital heart disease is among the most common cause of spontaneous abortion in humans. Developmental changes in systolic and diastolic ventricular function are required to meet the metabolic demand of the rapidly growing embryo. Ventricular filling during diastole is an important determinant of cardiac performance, and reflects the interaction of ventricular active relaxation, passive compliance, and filling load. Because disturbances in diastole often precede or occur independent from systolic cardiac failure, complete assessment of cardiac performance requires the ability to measure diastolic function. Others have reported previous attempts to measure diastolic function in chick and mouse embryos, However, they lack investigations into the relationship between ventricular diastolic function and myoarchitecture.

The developing ventricular myocardium is composed of compact and trabecular myocardium. Based on the spatial expression pattern of genes involved in conduction, contraction-relaxation, and calcium homeostasis, it has been speculated that the trabecular myocardium has faster contraction and slower relaxation than the compact myocardium, whereas the compact myocardium appears to be better adapted for ventricular relaxation. Recently, several mouse models with abnormal ventricular myoarchitecture have been generated by gene targeting. Diminished ventricular trabeculation has been reported in neuregulin and MEF2C mutant mice. Decreased myocardial compaction has been reported in FOG-2 (friend of GATA), Nf1, and RXR mutant mice. Analysis of ventricular systolic and diastolic dysfunction in mutants with selective deficiencies in trabecular or compact myocardium will provide additional insights into the interrelationships between genetic regulation, ventricular myoarchitecture, and systolic or diastolic ventricular function. However, early assessment of diastolic dysfunction in these gene-targeted models has been difficult because of a...
lack of normal murine data for diastolic function associated with cardiac morphogenesis. Therefore, we assessed ventricular diastolic as well as systolic function at multiple gestational ages and analyzed the correlation between diastolic function and myoarchitecture in normal mice. In addition, we applied our methods to FOG-2 mutant mice, which are embryonic lethal between embryonic day (ED) 12.5 to 15.5 with thin compact myocardium and tricuspid atresia.15,16 We show that FOG-2 mutant embryos have severely impaired diastolic function.

Materials and Methods

Animals
Data for normal mice was obtained using strain 129Sv. FOG-2 mice were in a mixed C57BL6/129Sv background. FOG-2 null embryos (FOG2KO) were compared with wild-type littermate controls (WT). DNA was isolated from embryo yolk sacs or tail biopsies and genotyped by polymerase chain reaction. All aspects of animal care and experiments performed in this study were approved by Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center.

Embryo Preparation for Hemodynamic Assessment
On ED 9.5 to 19.5, a pregnant mouse was anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and connected to a volume-cycled rodent ventilator (Harvard Apparatus model 683, Harvard Apparatus, South Natick, Mass). The rectal temperature of anesthetized mice was maintained at 37°C on a prewarmed heated platform. Part of the uterus containing one embryo was taken out and placed in a bath system perfused with oxygenated Hank’s balanced salt solution.3,4 Because heart rate (HR) is highly sensitive to temperature,19 the temperature of the bath was maintained at 37°C. The uterus wall and yolk sac were gently opened to expose the embryo. Umbilical vessels connecting the embryo with the placenta were kept intact. We could not obtain all hemodynamic data in the same embryo because of technical difficulties. Cardiac function was stably documented for more than 10 minutes in 315 of 420 embryos (75%).

Image Acquisition and Analysis of Cardiac Function
Images of cardiac contraction and relaxation were recorded with a color CCD camera mounted on a stereoscopic zoom microscope. Selected images were transferred online to a computer for ventricular size analysis. The outer ventricular (epicardial) border was manually traced from the frontal position and its area was measured by computer-assisted planimetry (IP laboratory, Scanalytics, Inc). Area ejection fraction (AEF) of the ventricle was calculated as follows:

\[ \text{AEF} = \frac{\text{EDV} - \text{ESV}}{\text{EDV}} \]

where \( \text{EDV} \) is end-diastolic ventricular volume, \( \text{ESV} \) is end-systolic ventricular volume, and \( \text{ESV} \) is corrected for body size. AEF was measured from the ventricles and the pressure was recorded with Acknowledge software (Figure 1). We measured atrial contraction (called atrial kick pressure) (a), end-diastolic pressure (EDP) (b), peak systolic pressure (c), and negative pressure by ventricular suction22,23 (called suction pressure) (d). Also, we calculated changes in pressure per unit time (dP/dt, –dP/dt).

Assessment of Ventricular Myoarchitecture
The ventricles were perfusion-fixed at high-flow low pressure25 with 4% paraformaldehyde to avoid variable contraction artifact, embedded into paraffin, and serial sections of 7 μm thickness were cut in the horizontal plane. The area of the myocardial layer was measured.
at the lateral free wall in both left ventricle (LV) and right ventricle (RV) using sections obtained at the level of major papillary muscles (Figure 2A, boxes). The compact region (Figure 2B, hatch pattern), the trabecular region (Figure 2B, stipple pattern), and the trabecular zone within lateral free wall (Figure 2B, the zone circled by a dotted line) were measured as relative developmental indices, and the area per 1 μm myocardium was calculated.

Statistical Analysis
Data are presented as mean±SD. Comparisons between two groups were analyzed with the Mann-Whitney U-test, for nonparametric distributions with unequal variances. Statistical significance was set at P<0.05. Partial correlation coefficients were calculated and regression analysis was performed among hemodynamic and morphological indices. Statistical significance among indices were judged by t test at P<0.05.

Results
Developmental Changes in Ventricular Myoarchitecture
The compact region in both ventricles started to increase at ED 12.5 after coronary arteries invaded the ventricular myocardium (Figure 3A). The trabecular zone in both ventricles increased rapidly from ED 9.5 to 13.5 and reached a maximum (Figure 3B, squares), whereas the trabecular region increased logarithmically from ED 9.5 to 19.5 (Figure 3B, squares).
circles), so that the density of trabeculae, measured as the ratio of trabecular region to zone, increased from 0.37±0.01 at ED 10.5 to 0.98±0.03 at ED 19.5 in LV (Figure 3C, open circles), and from 0.40±0.02 to 0.75±0.02 in RV (Figure 3C, closed circles).

Developmental Changes in Ventricular Size and Function
Table 1 shows the changes in ventricular EDA and AEF from ED10.5 to 14.5. At ED 11.5, the size of right and left ventricles was the same, 0.77±0.07 mm². At ED 12.5, LVEDA reached a maximum 0.80±0.02 mm². At ED 14.5, RVEDA increased to 1.29±0.10 mm², 59% more than LVEDA. AEF did not change significantly during these early embryonic stages.

Developmental Changes in Pulsed Doppler Blood Velocity
Compared with the adult heart, the ventricular filling pattern in the embryonic heart was characterized by relatively higher peak A velocity than peak E velocity (for example, see Figure 6A). Figure 3D shows developmental changes in peak E velocity at each gestational age. Ventricular active relaxation, evaluated by peak E velocity, increased exponentially from ED 9.5 to 19.5. Figure 3E shows developmental changes in peak A velocity. Ventricular passive compliance, evaluated by peak A velocity, decreased logarithmically from ED 9.5 to 19.5. The E/A ratio decreased after ED 9.5 to reach nadir between ED 12.5 to 13.5, and subsequently the E/A ratio increased with development (Figure 3F).

Developmental Changes in Ventricular Pressure
Figure 1 shows representative ventricular pressure waveforms from ED 10.5 to 13.5. Embryos had higher EDP and atrial kick pressure relative to a peak pressure than adult mice. Table 2 summarizes developmental changes in ventricular pressure for ED 9.5 to 14.5 mouse embryos. Peak systolic pressure increased significantly from 2.13±0.36 mm Hg at ED 9.5 to 11.15±0.54 mm Hg at ED 14.5 (P<0.05), whereas EDP increased significantly from 0.43±0.10 to 0.88±0.08 mm Hg (P<0.05).

Correlation Between Ventricular Diastolic Function and Myoarchitecture With Development
The partial correlation coefficients (r) between peak E and A velocities, compact region, trabecular region, trabecular zone, and HR were calculated. Statistical analysis of partial correlation coefficients by t test showed that the correlation between peak A velocity and trabecular region was significant in both ventricles (LV, r²=0.52; RV, r²=0.38; P<0.05). Similarly, the correlation between peak E velocity and compact region was significant in both ventricles (LV, r²=0.76; RV, r²=0.82; P<0.05). The correlation between peak A velocity and HR was also significant (LV, r²=0.41; RV, r²=0.50; P<0.05).

Figure 4 shows the results of regression analysis between peak E velocity and compact region in LV (Figure 4A) and RV (Figure 4B), and between peak A velocity and trabecular region in LV (Figure 4C) and RV (Figure 4D). Peak E velocities in both ventricles correlated well with compact region (in LV, y=14.58 e^{0.013x}, r²=0.97, P<0.0001, in RV, y=13.53 e^{0.015x}, r²=0.98, P<0.0001).
Diastolic Dysfunction in FOG-2 Mutant Mouse

To extend our methods for analyzing myoarchitecture and hemodynamics to mutant embryos, we elected to study FOG-2 null embryos, because we hypothesized based on the thin compact myocardium of these embryos that they would have diastolic dysfunction. At ED 13.5, compact region in FOG2KO was smaller than that in WT both in LV and RV (18±4 (n=5) versus 38±4 μm²/μm (n=5), P<0.05, in LV, and 17±1 (n=5) versus 45±4 μm²/μm (n=5), P<0.05, in RV). Trabecular region in FOG2KO was also smaller than that in WT both in LV and RV (40±4 (n=5) versus 99±6 μm²/μm (n=5), P<0.05, in LV, and 38±3 (n=5) versus 88±4 μm²/μm (n=5), P<0.05, in RV), whereas there was no significant difference of trabecular zone between FOG2KO and WT both in LV and RV (data not shown). The density of trabeculae was lower in FOG2KO than in WT (0.27±0.02 versus 0.64±0.04, P<0.05, in LV, and 0.26±0.02 versus 0.57±0.04, P<0.05, in RV).

At ED13.5, there was no significant difference in AEF in either LV or RV between FOG2KO and WT (data not shown). Right ventricular EDA in FOG2KO was significantly smaller than in WT embryo (1.01±0.04 (n=8) versus 1.16±0.05 mm² (n=13), P<0.05), whereas there was no significant difference in left ventricular EDA between FOG2KO and WT (data not shown). There was no significant difference in HR between FOG2KO and WT (data not shown).

Figure 5 shows a comparison of ventricular pressure data from FOG2KO and WT at ED13.5. There were no significant differences in peak ventricular pressure (Figure 5C), atrial kick pressure (Figure 5E), or dP/dt (Figure 5G) between FOG2KO and WT. On the other hand, EDP in FOG2KO was significantly higher than in WT (1.6±0.2 versus 0.8±0.1 mm Hg, P<0.05) (Figure 5D); sucking pressure in FOG2KO mutant was diminished compared with WT (0.2±0.2 versus −0.6±0.1 mm Hg, P<0.05) (Figure 5F); and −dP/dt in FOG2KO was significantly smaller than in WT (170.0±10.8 versus 191.9±13.3 mm Hg/sec; P<0.05) (Figure 5H).

Figure 6 shows the comparison of ventricular inflow velocity data from FOG2KO and WT at ED13.5. The E wave in FOG2KO was significantly higher than in WT (57.0±5.7 versus 24.4±1.7 mm/sec; P<0.01) (Figure 6C), whereas there was no significant difference in the A wave between FOG2KO and WT (91.0±4.2 versus 96.1±6.0 mm/sec; NS) (Figure 6D). The E/A ratio in FOG2KO was significantly larger than in WT (0.63±0.05 versus 0.25±0.02; P<0.05) (Figure 6E).

An abnormal regurgitant blood flow during ventricular systole was observed in the inferior vena cava of FOG-2 null embryos at ED 13.5 (data not shown), indicating that the FOG-2 mutant heart had atrioventricular (AV) valve regurgitation.

Discussion

Developmental Changes in Diastolic Properties of Mouse Embryonic Myocardium

Our results are consistent with previously reported results in rat, chick, and human that showed that mice embryonic myocardium is stiffer than mature adult myocardium. Lower E and higher A wave velocity in embryonic heart compared with adult heart indicate a stiffer ventricular myocardium in embryo. Although the ventricular filling pattern can be influenced by many factors other than ventricular diastolic function (see Limitations section), the E/A ratio have been considered to be useful to compare developmental changes of ventricular diastolic function in normal mouse. In addition, this conclusion is independently supported by our direct intraventricular pressure measurements. Relative to peak...
ventricular pressure, EDP and atrial kick pressure were higher in embryonic ventricles than in mature adult mouse ventricles.26 These hemodynamic results are compatible with biomechanical and morphological findings previously reported in sheep,31 human,32 and chick embryo,33 although interspecies differences may limit the extrapolation of these results to mice.

Based on the analysis of E/A ratio, the embryonic myocardium is maximally stiff at ED 12.5 to 13.5. The exponential increase in peak E velocity indicates that ventricular active relaxation develops exponentially during embryonic development, whereas the logarithmic increase in peak A velocity indicates that ventricular passive compliance decreases in early embryonic stages and does not change significantly throughout late embryonic stages. These results are compatible with previous reports in human23 and mouse embryos.10

Correlation of Developmental Changes in Diastolic Function and Myoarchitecture

Ventricular diastolic filling is influenced by various factors, including diastolic filling time,7,34 ventricular geometry,35 and ventricular material properties.36 It has been demonstrated that ventricular filling characteristics correlate with gestational age and HR in the human fetus23 and chick embryo.7 Also, it has been shown that during acute changes in HR, the chick embryo maintained cardiac output by a compensatory change in stroke volume.34

Another important factor that has been suggested to influence ventricular diastolic function is ventricular myoarchitecture. Our statistical analysis shows that ventricular active relaxation in mouse embryo exponentially correlates with compact region (Figures 2B, 4A, and 4B). Compared with trabecular myocardium, the compact myocardium expresses higher levels of alkali ventricular myosin light chain (MLC1V), regulatory ventricular myosin light chain (MLC2V), connexin 43, sarcoplasmic Ca$^{2+}$-ATPase 2A (SERCA2A), and phospholamban (PLB), suggesting that the compact myocardium is better adapted to relaxation than trabecular myocardium.11,12 The exponential correlation between active relaxation and compact region may be due to developmental changes in the properties of the compact myocardium, including changes in both myofilament and nonmyofilament structures and processes, such as the extramyofilament cytoskeleton33,37,38 and calcium metabolism.39,40 Myofibril never completely fills the myocyte in early embryonic stage and the lack of myofibrillar organization might affect systolic and diastolic myocardial function.33,37,38 The genes encoding the calcium regulatory proteins, SERCA2A, PLB, and sarcolemmal Na$^+$-Ca$^{2+}$ exchanger (NCX1), are expressed at barely detectable levels before ED12.5, and increase during mid and late
Both developmental organization of myofibrils and developmentally regulated increases in the expression of these calcium regulatory protein genes likely result in progressive increases in the rate of relaxation of compact myocardium.

On the other hand, our data indicate that ventricular passive compliance mainly correlates with trabecular region (Figures 2B, 4C, and 4D). Compared with compact myocardium, trabecular myocardium expresses higher levels of α-myosin heavy chain (α-MHC), alkali atrial myosin light chain (MLC1A), regulatory atrial myosin light chain (MLC2A), and connexin 40, whereas it expresses lower levels of SERCA2A and PLB, suggesting that trabecular myocardium is suitable for faster contraction and has slower relaxation compared with compact myocardium. The close association of the developing His-Purkinje system with the trabecular myocardium may also facilitate faster contraction by trabecular compared with compact myocardium. Because of the geometry of the ventricle, the trabecular myocardium occupies the inner side and thus is mechanically less stretched in diastole than the outer side. In addition, the spongiform nature of trabecular myocardium possibly has a higher passive compliance than compact myocardium. This combination of factors may account for the correlation between trabecular region and ventricular compliance at different gestational ages.

**Diastolic Dysfunction in FOG-2 Mutant Mice**

We show that FOG-2 null embryos have impaired ventricular diastolic function with underdeveloped compact myocardium and trabecular density. At ED 13.5, FOG-2 null embryos have preserved systolic function, as measured by ventricular peak pressure, +dP/dt, and AEF. In contrast, diastolic function is severely impaired, as reflected by higher EDP and diminished sucking pressure and −dP/dt in FOG2KO compared with WT. This conclusion is independently corroborated by Doppler velocimetry data, which show a higher E wave and E/A ratio in FOG2KO compared with WT. This ventricular inflow pattern can be seen in the setting of restrictive ventricular filling with elevated atrial pressure.

Doppler assessment of inferior vena cava flow demonstrated retrograde flow during ventricular systole in FOG-2 null embryos, indicative of AV valve regurgitation. AV valve regurgitation and elevated EDP suggest elevated atrial pressure in FOG-2 null embryos; atrial dilatation in these embryos is likely a consequence of elevated atrial pressure. AV valve regurgitation can confound the assessment of diastolic dysfunction by Doppler measurement of inflow velocity. However, it should not alter parameters of diastolic function derived from intraventricular pressure measurements, and therefore, we feel our diagnosis of diastolic dysfunction in these embryos is valid even in the presence of AV valve regurgitation.

AV valve regurgitation, observed in FOG-2 null mutant heart, usually causes enlarged atrium, hyperkinetic ventricular contraction, and ventricular dilatation due to volume overload. However, in FOG-2 mutants at ED 13.5, the LV is not dilated in spite of a markedly enlarged atrium, indicating that ventricular distensibility might be impaired. The smaller RV in FOG-2 mutants at ED 13.5 might be caused by the effect of tricuspid atresia-like hemodynamics. As a result of AV valve regurgitation and the failure of myocardial compensation for volume overload due to decreased ventricular distensibility, cardiac output in FOG-2 mutant embryos should be reduced, resulting in embryonic growth retardation and lethality.

**Limitations**

We measured ventricular inflow velocity to assess ventricular diastolic function. Although the ventricular filling pattern can be influenced by many factors other than ventricular diastolic function, such as changes of preload, atrial function, and HR, E, A, and E/A have been considered to be useful to compare developmental changes of ventricular diastolic function in normal mouse. The peak A velocity is not an absolute measure of ventricular passive compliance; however, ventricular passive compliance is an important determinant of the peak A velocity. This allows the peak A velocity to be used to make relative comparisons between normal and abnormal hemodynamics and between normal embryos at different gestational ages. This method has become popular for the clinical evaluation of ventricular diastolic function in the human fetus. Importantly, our interpretation of ventricular filling pattern is independently supported by our direct intraventricular pressure measurements, including EDP, sucking pressure, and −dP/dt.

We used an invasive method that allowed the direct visualization of cardiac contraction and relaxation, and the measurement of ventricular pressure. Although the advantages of this invasive approach make it well suited for future studies of embryonic cardiac function in genetically manipulated mice, there are important limitations. As with all invasive methods, hemodynamics may be altered as a result of the requisite experimental manipulations. However, we do not believe this was a significant problem, because our data are largely consistent with those from other groups, including those using noninvasive methods. In one of the noninvasive studies, 30% to 50% higher HR than those in the other studies has been reported.

We showed that ventricular sucking pressure was useful to detect ventricular diastolic dysfunction in FOG-2 mutant mice. Although the frequency response characteristics of the thin pipettes used for intraventricular pressure measurements might technically limit our ability to detect small, transient changes of ventricular pressure, we confirmed the reproducibility of negative sucking pressure measurements in normal embryos, and we could clearly distinguish FOG-2 mutant from WT embryos based on the more positive sucking pressure values in FOG-2 null embryos compared with WT. The ventricular pressure in chick embryo has been reported to fall to negative values during early diastole, using the same servo-null micropressure system that we used. Therefore, we believe that the measurement of sucking pressure by our micropressure system is sufficiently accurate and reproducible for it to be...
useful to compare ventricular diastolic function between embryos.

Finally, significant differences in hemodynamics are known to exist between mouse strains. We did not see any statistical difference of cardiac function between normal data obtained in 129Sv mice and WT data obtained in a mixed C57BL/6J/129Sv background (data not shown). Because there may be some differences of diastolic function in embryonic heart in other strains, further studies may be required to account for the effect of strain background on measurements of diastolic function in other genetically modified mice.

Conclusions

We propose that both the maturation and the proliferation of compact myocardium determine ventricular active relaxation, whereas proliferation of trabecular myocardium correlates with ventricular passive compliance. Our methods and data on normal embryo physiology form the basis for future studies of embryonic cardiac function in genetically manipulated mice with abnormalities of the cardiovascular system. This is illustrated by our hemodynamic analysis of FOG-2 mutant mice, in which systolic function exponentially manipulated mice with abnormalities of the cardio-myocardial neuregulin receptor. Nature. 1995;378:390–394.

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