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The effects of reduced hemodynamic loading on morphogenesis of the mouse embryonic heart

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ABSTRACT

Development of the embryonic heart involves an intricate network of biochemical and genetic cues to ensure its proper growth and morphogenesis. However, studies from avian and teleost models reveal that biomechanical force, namely hemodynamic loading (blood pressure and shear stress), plays a significant role in regulating heart development. To study how hemodynamic loading impacts development of the mammalian embryonic heart, we utilized mouse embryo culture and manipulation techniques and performed optical projection tomography imaging followed by morphometric analysis to determine how reduced-loading affects heart volume, myocardial thickness, trabeculation and looping. Our results reveal that hemodynamic loading can regulate these features at different thresholds. Intermediate levels of hemodynamic loading are sufficient to promote proper myocardial growth and heart size, but insufficient to promote looping and trabeculation. Whereas, low levels of hemodynamic loading fails to promote proper growth of the myocardium and heart size. These results reveal that the regulation of heart development by biomechanical force is conserved across many vertebrate classes, and this study begins to elucidate how these specific forces regulate development of the mammalian heart.

1. Introduction

Morphogenesis of the heart is a complex process involving both genetic, biochemical and biomechanical cues to ensure that the heart undergoes rightward looping, chamber growth, myocardial thickening, trabeculation, valvulogenesis, and septation. Though our understanding of the control of these processes has been clarified by genetic studies, we are learning more about the role that biomechanical forces play in cardiac morphogenesis (Bajolle et al., 2009; Culver and Dickinson, 2010; Midgett and Rugonyi, 2014).

Several studies have shown that manipulation of hemodynamic loading (blood pressure and shear stress) leads to an impairment in heart development. Many of these studies rely heavily on zebrafish and chicken embryo manipulation techniques to alter blood flow or blood pressure. For instance, hemodynamic loading can be altered in zebrafish embryos by occluding the outflow and inflow tracts with glass beads (Hove et al., 2003), by genetic manipulation to alter blood flow patterns or amount (Heckel et al., 2015; Yashiro et al., 2007), by drug treatment to impair contractions (Yang et al., 2014), or by centrifugation of the embryos (Johnson et al., 2015). In chicken embryos, manipulations are performed by vitelline vein ligations (to reduce hemodynamic loading), atrial clipping (to increase hemodynamic loading of the ventricles), conotruncal banding (Broekhuizen et al., 1999; deAlmeida et al., 2007; Midgett and Rugonyi, 2014; Stekelenburg-de Vos et al., 2005, 2007), by laser ablation of regions of the heart to impair site-specific heart contractions (Yalcin et al., 2010), and by using pulsed infrared light to alter pacing of the heart (Jenkins et al., 2010). These manipulations result in a wide range of cardiac abnormalities, some of which mimic human congenital heart defects.

In mice, much less is studied about the impact of hemodynamic loading on heart development because of the challenge in culturing and manipulating the embryo. Thus, most studies rely on mutations in genes that affect heart contraction, which in turn diminishes hemodynamic loading, such as *Myl7* (*Mlc2a*) (Huang et al., 2003), the *Cacnb2* (*CaVbeta2*) (Weissgerber et al., 2006), and *Titin* (*Ttn*) (May et al., 2004), and *Slc8a1* (*Ncx1*) (Wakimoto et al., 2000). Though mutations

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in these genes do result in impaired heart development, determining which abnormalities are the result of a direct defect to the heart, or an indirect defect caused by altered blood flow, is difficult to elucidate.

In this study, we sought to determine whether reductions in hemodynamic loading can alter mouse heart development. Our study uses a previously established technique to reduce hemodynamic force in the cardiovascular system by the production and culturing of low-hematocrit mouse embryos (Lucitti et al., 2007). Low-hematocrit embryos can be made by injecting acrylamide and TEMED (AT) into the yolk sac blood island vessels. These agents polymerize to prevent all blood cells from entering circulation, resulting in a reduction of blood viscosity in circulation and therefore a reduction in hemodynamic loading. Thus, in this study, we refer to the low-hematocrit embryos as "reduced-loading embryos". Using the reduced-loading embryos, we compared several morphological characteristics of the E9.5 mouse heart to control embryos. Embryos were imaged by Optical Projection Tomography (OPT) microscopy to obtain high-quality 3D images, and morphometric analysis was performed to statistically determine if there were changes in several features of heart development: heart volume, myocardial thickness, trabeculation and looping. Our results revealed that reducing the hemodynamic load resulted in a decrease in heart volume and myocardial thickness. Further, only a slight decrease in hemodynamic loading (an unanticipated consequence of acrylamide treatment alone) was needed to impair trabeculation and looping. Collectively, this study reveals that mammalian systems do require normal hemodynamic loading conditions for proper development of the heart.

2. Materials and methods

2.1. Mice

CD1 mice (*Mus musculus*) were used for the embryo culture/manipulation studies, and to assess developmental delay. *Mlc2a/Myl7* mutant mice were also used for the developmental delay study (Huang et al., 2003). Mice were cared for under the guidelines of IACUC-approved vertebrate animal protocols (15–004.0 and 15–008.0).

2.2. Production and culturing of reduced-loading embryos

Embryonic day (E) 8.5 mouse (*Mus musculus*) embryos were dissected out of the uterus, and the decidua and parietal yolk sacs were removed (with visceral yolk sac and embryo intact). Isolated embryos were cultured statically, as previously published (Udan and Dickinson, 2010). Briefly, embryos were dissected in dissection medium (DMEM/F12 [Gibco® by Life Technologies™, Cat# 11330-032] 45mls, 5 mls fetal bovine serum [Gibco™, Cat # 26140079], and 0.5 mls of 100 × penicillin/streptomycin [Sigma, Cat# P4458–100 ml]) that has been equilibrated in a tissue culture incubator (maintained at 37 °C, 5% CO_2) for 1 h. Embryos were injected with various substances into the blood islands region of the yolk sac vasculature (see below) and placed back in the tissue culture incubator for 30 min to equilibrate. After incubating, the embryos were transferred to culture medium (1:11 ml of dissection medium: rat serum [Rat Serum, Valley Biomedical, #AS3061], medium initially exposed to 5% CO_2, 20% O_2, and balanced N_2 for 4 min).

The procedure for making reduced-loading/low-hematocrit embryos was performed as previously published (Lucitti et al., 2007). After dissecting embryos out of the uterus, they were allowed to equilibrate for 30 min to 1 h in dissection medium. After equilibrating, 3–6 somite stage embryos were selected for injection. This range was used because embryos that were too young (before 3 somites) did not have fully lumenized vessels, so they could not be injected and were thus not used. Conversely, embryos that were too old (after 6 somites) were also discarded because blood has already entered into circulation, and thus the blood cells could not be immobilized. The remaining 3–6 somite stage embryos were parsed out into control and experimental groups, having evenly matched somites in both groups. Control groups were either not-injected (NI control embryos), or injected with substances in the blood island vessels that should not affect hematocrit levels (A_O = acrylamide only, T_O = TEMED only, P_O = PBS only). Experimental embryos were injected with both Acrylamide and TEMED (AT), and these were referred to as reduced-loading embryos or AT embryos. AT embryos used in the study resulted in all blood cells failing to enter circulation, similar to what has been previously published (Lucitti et al., 2007). Injections were performed using glass-pulled needles (World precision instruments, Cat #: PMP-102) and a picoinjector (Warner Instruments, Cat. # PLI-10). For all injections, India ink (American MasterTech, Cat. # STIIN25) was used to track presence of the injected bolus.

2.3. Developmental delay

After 24 h of culture, somites for experimental and control embryos were counted under the dissection microscope. For those embryos that were imaged, accuracy of the counts was verified by also counting somites from 3D images of embryos produced by OPT microscopy (see below).

2.4. Immunostaining and clearing

Control and experimental embryos after 24 h of culturing were immunostained using standard immunostaining procedures. Embryos were fixed in 4% paraformaldehyde for 1 h, washed in PBS, blocked in blocking buffer (PBS, 0.8% Triton X, 5% normal donkey serum, 1% BSA) for 1 h, washed in SBT (PBS, 0.8% Triton-X, 2% normal donkey serum) buffer, blocked in blocking buffer (PBS, 0.8% Triton-X, 5% normal donkey serum and 1% BSA) and incubated with anti-Actin α-Smooth Muscle antibody, conjugated to the Cy3 fluorophore (Sigma-Aldrich, Cat# 6198-0.2 ml) in SBT buffer. After incubation, embryos were then washed with 1 × PBT, stored in PBS with 1% sodium azide at 4 °C. Before clearing the embryos, yolk sacs were removed and somites were counted.

To clear the embryos and prepare them for OPT microscopy, embryos were immersed into 1% agarose in PBS, and aspirated into a transfer pipet cut off at the tip (Thermo Scientific™, Catalog # 2321S). The transfer pipet was placed horizontally and rolled around to ensure the embryo remained in the center of the agarose cylinder. After cooling, the agarose cylinder with the embryo inside was removed, and placed in a dehydrating solution of 25% methanol in PBS, and incubated for 24 h. Every 24 h, the solution was changed out for increasing amounts of methanol: 50%, 75%, and 100% (2×). Next, the embedded embryos were cleared in a 1:2 solution of Benzyl Alcohol (Fisher, Cat# A396-500): Benzyl Benzoate (Acros Organics, Cat# CAS 120-51-4) (BABB) for 24 h. The next day, a fresh solution of BABB was replaced, and embryos were stored until they were ready to be imaged. Embryos were considered cleared when neither the embryo nor the agarose could be seen by eye in the BABB solution.

2.5. OPT imaging

Embryos were imaged using a custom-made Optical Projection Tomography (OPT) microscope (Singh et al., 2015; Wong et al., 2013). The agarose embedded embryos were glued onto a metal chuck, which was then magnetically suspended onto a motorized and inverted stage. The motorized stage was lowered to immerse the embedded embryo into a glass cuvette containing BABB. The sample was aligned at the focal plane, and positioned in a manner to keep it centered during the sample rotation process. To detect the heart labeled with Sma-Cy3 staining, excitation light (BP 531/40 filter) was sent to the embryo at an angle away from the camera. Fluorescent light from the embryo was transmitted through an emission filter (BP 593/40), magnified by the