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The endoderm and myocardium join forces to drive early heart tube assembly

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ABSTRACT

Formation of the muscular layer of the heart, the myocardium, involves the medial movement of bilateral progenitor fields; driven primarily by shortening of the endoderm during foregut formation. Using a combination of time-lapse imaging, microsurgical perturbations and computational modeling, we show that the speed of the medial-ward movement of the myocardial progenitors is similar, but not identical to that of the adjacent endoderm. Further, the extracellular matrix microenvironment separating the two germ layers also moves with the myocardium, indicating that collective tissue motion and not cell migration drives tubular heart assembly. Importantly, as myocardial cells approach the midline, they perform distinct anterior-directed movements relative to the endoderm. Based on the analysis of microincision experiments and computational models, we propose two characteristic, autonomous morphogenetic activities within the early myocardium: 1) an active contraction of the medial portion of the heart field and 2) curling- the tendency of the unconstrained myocardial tissue to form a spherical surface with a concave ventral side. In the intact embryo, these deformations are constrained by the endoderm and the adjacent mesoderm, nevertheless the corresponding mechanical stresses contribute to the proper positioning of myocardial primordia.

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Introduction

In amniotes, the endoderm plays an important mechanical and signaling role in early heart development. As cardiac precursors in the splanchnic mesoderm move from bilateral heart fields to the embryonic midline, they are in close contact with the endoderm and intervening extracellular matrix. Myocardial precursor cells become epithelialized beginning at Hamburger and Hamilton (1951)/HH Stages 7-8 (Linask, 1992), and the myocardial tissue primordia fuse anterior to the opening of the foregut—the anterior intestinal portal (AIP) at HH9-10. The foregut lining is formed concomitantly via shortening of the endoderm, and the walls of the foregut are formed from splanchnic mesoderm adjacent to the myocardium (Bellairs and Osmond, 1998). The physical association between the mesoderm and endoderm (connected by extracellular matrix), as well as the forming heart and foregut (connected transiently via a mesentery) indicates the need to study tissue-level mechanical interactions during heart and foregut formation.

In avians, several studies have suggested that myocardial progenitors actively propel the midline-directed movement of cardiogenic fields (DeHaan, 1963; Rosenquist and DeHaan, 1966; Wiens, 1996). Currently the most widely accepted (textbook) mechanism that attempts to explain these movements is the *migration* of myocardial cells (Gilbert, 2006). This migration is envisioned as taking place relative to the underlying endoderm of the forming foregut and the associated extracellular matrix (ECM). Furthermore, timely closure (regression) of the anterior intestinal portal (AIP) is critical for the midline directed myocardial precursor movements (reviewed in Brand (2003)), as perturbation of AIP regression or removal of the foregut endoderm results in cardia bifida (DeHaan, 1959; Rosenquist, 1970; Gannon and Bader, 1995). Varner and Taber (2012) provided additional evidence for a primary role of endoderm shortening (contraction) in driving convergence of the heart fields to the midline, and co-movement of labeled endodermal and myocardial tissue was demonstrated. In this study, we sought to determine if shortening of the endoderm was sufficient to form a tubular heart, or whether myocardial progenitors actively participated in driving the fusion of myocardial progenitor fields at the midline.

We demonstrate that in avians, myocardial precursors do not migrate substantially relative to their ECM microenvironment as

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has been suggested. Instead, in agreement with the results of Varner and Taber (2012), endodermal shortening during foregut morphogenesis predominantly drives the medial-ward displacement of the myocardial cells to the midline. However, here we show that in addition to the role of the endoderm-as the myocardial progenitor fields are moving towards the midline-they autonomously exert mechanical stresses within the tissue. These forces give rise to at least two distinct *active* autonomous deformations and propel the anterior displacement of the myocardium relative to the endoderm. Thus, our imaging and microincision studies as well as our computational models indicate that both endodermal contraction and autonomous myocardial deformations contribute to heart tube assembly.

Materials and methods

Quail embryo preparation

Fertile wild type quail (*Coturnix coturnix japonica*) eggs (Ozark Egg Co., Stover, MO) were incubated for varying periods of time (from 20 to 36 h) at 37 °C to reach stages HH6 to HH11 (Hamburger and Hamilton, 1951). Embryos were then isolated and cultured as in Cui et al. (2009) (modified early chick "EC" culture; Chapman et al., 2001).

Plasmid generation and transfection for myocardial progenitor labeling

Genomic DNA was prepared from three HH12 chick embryos using Tri Reagent (Sigma) according to the manufacturer's instructions. Primer sequences 5′ -AAAAAATTAATCAAGGCTATGACTGCTGGAGTG-3′ and 5′-AAAAAGCTAGCCAGAGGTGCTGGTGGTGCTG-3′ were used to PCR amplify the region between −931 and +56 of the Cardiac Myosin Light Chain 2 (CMLC2/my17) gene. To generate pCMLC2::EGFP plasmid, the CMLC2 promoter was subcloned into the pEGFP-C1 vector (Clontech) between AseI and NheI restriction sites in place of the CMV promoter. To generate pCMLC2::MitoRFP, the CMLC2 promoter was similarly subcloned into the pEGFP-N1 vector (Clontech) between AseI and NheI sites. Next, the EGFP ORF was replaced by a cassette consisting of a mitochondrial localization signal fused to RFP (MitoRFP) using XhoI and NheI restriction sites. The MitoRFP cassette was derived from pCoxIV-RFP (a generous gift from Dr. Rusty Lansford, University of Southern California and Children's Hospital Los Angeles, CA).

In vivo fluorescent labeling approaches

Monoclonal antibodies directed against fibrillin-2 (JB3) and fibronectin (B3D6) ECM proteins (Rongish et al., 1998, Czirok et al., 2006, Aleksandrova et al., 2012; DSHB, Iowa City, IA) or a quail endothelial cell surface marker (QH1; Pardanaud et al., 1987; DSHB) were directly conjugated to AlexaFluor 488, 555 or 647 (Molecular Probes) according to the manufacturer's instructions. The direct conjugates were injected into the lateral plate mesoderm in 5–40 nl boluses using a PLI-100 (Harvard Instruments) microinjector as described in Little and Drake (2000). Microinjections were performed 30–60 min prior to the beginning of the image acquisition to allow for antibody diffusion and antigen binding. We found that short exposure of the ventral side of the embryo to the MitoTracker Green dye solution, a dye retained in mitochondria, causes its retention only in the endoderm, if endodermal integrity was not compromised during treatment. The in vivo endoderm-labeling MitoTracker® Green FM dye (Invitrogen) was diluted in DMSO to 1 mM concentration according to the manufacturer's recommendations. The obtained stock solution was further dissolved to 500 nM in PBS. HH8 stage embryos, placed on paper rings as described above, were submerged into the resultant solution with their ventral side down, and placed into a humidified incubator at 37 °C for 30 min. Following three 5 min washes with PBS at room temperature, the quality of labeling and the integrity of the endoderm in each embryo was assessed under a Leica DM6000 epifluorescence microscope.

Post-fixation immunofluorescence labeling

Embryos were fixed and prepared for immunolabeling according to Little and Drake (2000). Monoclonal antibodies against avian epitopes (JB3, B3D6, QH1; DSHB, Iowa City, IA), directly conjugated to AlexaFluor 488, 555 or 647 (Molecular Probes, Eugene, OR), were added at 1:1000 dilutions; rabbit polyclonal anti-GFP antibody, conjugated to AlexaFluor 488 – at 1:400, and mouse monoclonal antibody MF20 (DSHB, Iowa City, IA) – at 1:10 dilution in 3% BSA for overnight incubation. Goat anti-mouse secondary antibodies, conjugated to AlexaFluor 488, 555 or 647, were used to follow the MF20 staining at 1:10 dilution in 3% BSA. Nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) at 500 nM in PBS.

Preparation of transverse plastic sections

Embryos were dehydrated through a graded ethanol series, placed in JB4 infiltration medium (Electron Microscopy Sciences, Hatfield, PA) at 4 °C overnight, and embedded in JB4 resin following the manufacturer's protocol. Subsequently, 10 μm sections were prepared.

Wide-field time-lapse imaging

Automated microscopy of immunolabeled quail embryos was performed as described elsewhere (Czirók et al., 2002; Zamir et al., 2008). To enhance contrast, selected epifluorescence image stacks were deconvolved (Autoquant X, MediaCybernetics). Manual tracing of image details was performed using custom software (see, e.g., Czirok et al., 2004). Embryos stained with MitoTracker Green (as described above) were subsequently recorded in time lapse for 4–5 h with 10–12 min intervals between frames. Fading of MitoTracker Green fluorescent signal prevented further imaging. Motion analysis of recorded image sequences were performed as described below and in Aleksandrova et al. (2012).

Confocal imaging

Confocal imaging of whole-mount embryo specimens was performed using a Nikon 90i upright microscope with a Nikon C1 confocal scan head and Nikon Plan Apochromat 10× and 20× objectives.

Microincisions

50–800 μm incisions through the endoderm and adjacent splanchnic mesoderm of HH8 stage embryos were performed under a Leica stereomicroscope using tungsten needles. Embryos were then incubated at 37 °C on a microscope stage (Czirók et al., 2002; Zamir et al., 2008) for 0.5–1.5 h prior to initiation of time-lapse image acquisition.

Particle image velocimetry (PIV)

We used the two-step algorithm of Zamir et al. (2005); implemented in MatLab (Mathworks, Inc.) and as described in Aleksandrova et al. (2012). Briefly, images were divided into overlapping tiles, each 75 μm wide. The displacements of the tiles were determined by cross-correlation analysis; for each tile we searched the next image for the

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