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Multiple influences of blood flow on cardiomyocyte hypertrophy in the embryonic zebrafish heart

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ABSTRACT

Cardiomyocyte hypertrophy is a complex cellular behavior involving coordination of cell size expansion and myofibril content increase. Here, we investigate the contribution of cardiomyocyte hypertrophy to cardiac chamber emergence, the process during which the primitive heart tube transforms into morphologically distinct chambers and increases its contractile strength. Focusing on the emergence of the zebrafish ventricle, we observed trends toward increased cell surface area and myofibril content. To examine the extent to which these trends reflect coordinated hypertrophy of individual ventricular cardiomyocytes, we developed a method for tracking cell surface area changes and myofibril dynamics in live embryos. Our data reveal a previously unappreciated heterogeneity of ventricular cardiomyocyte behavior during chamber emergence: although cardiomyocyte hypertrophy was prevalent, many cells did not increase their surface area or myofibril content during the observed timeframe. Despite the heterogeneity of cell behavior, we often found hypertrophic cells neighboring each other. Next, we examined the impact of blood flow on the regulation of cardiomyocyte behavior during this phase of development. When blood flow through the ventricle was reduced, cell surface area expansion and myofibril content increase were both dampened, and the behavior of neighboring cells did not seem coordinated. Together, our studies suggest a model in which hemodynamic forces have multiple influences on cardiac chamber emergence: promoting both cardiomyocyte enlargement and myofibril maturation, enhancing the extent of cardiomyocyte hypertrophy, and facilitating the coordination of neighboring cell behaviors.

Introduction

The embryonic vertebrate heart undergoes a substantial morphogenetic transformation as it transitions from a simple heart tube to a series of bulbous chambers (Auman et al., 2007; Christoffels et al., 2004; Harvey, 2002). Asymmetric looping twists the linear heart tube into an S-shaped configuration that creates morphological distinction between the primitive chambers. At the same time, chamber volume enlarges through a process called ballooning, which results in the outer curvature of each chamber bulging out of the heart tube. During this process of chamber emergence, the developing heart also enhances its contractility. In chick, for instance, the speed of blood flow increases over 20-fold as the chambers form (Dunnigan et al., 1987; McQuinn et al., 2007). The proper execution of these morphological and functional transitions is essential to support the increasing physiological demands of the growing embryo; however, little is known about the cellular mechanisms underlying this transformation of the developing heart.

Several types of cell behaviors are likely to contribute to the process of chamber emergence. Both cardiomyocyte proliferation and cell size increase can help to facilitate chamber expansion. In chick, for example, cardiomyocyte proliferation is estimated to account for two-thirds of the overall chamber size increase during chamber formation (Soufan et al., 2006). The remainder of the chamber size increase is thought to result from increases in the size of individual cardiomyocytes, particularly in the region of the bulging outer curvature (Soufan et al., 2006). In zebrafish, our previous studies have shown an analogous regional increase in cardiomyocyte size at the outer curvature of the emerging ventricle (Auman et al., 2007). Cardiomyocyte size increase has also been observed in mouse embryos, where it has been noted that cardiomyocyte enlargement progresses throughout the course of embryonic heart development, accompanied by continually increasing maturation of myofibrils (Hirschy et al., 2006). The parallel augmentation of both the size and myofibril content of cardiomyocytes is often referred to as hypertrophic growth (Frey and Olson, 2003). It is appealing to consider that the uniform and coordinated execution of hypertrophic growth could play an important role in promoting the morphological and

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functional maturation of the cardiac chambers. However, it is not yet clear to what extent the hypertrophic growth of individual ventricular cardiomyocytes is coupled with the dynamic transformation of the heart tube.

Cultured cardiomyocytes robustly display hypertrophic growth when stretched (Russell et al., 2010; Yu and Russell, 2005), suggesting that hypertrophic growth taking place in the embryonic heart could be triggered by biomechanical forces. Embryonic circulation is initiated as soon as the heart tube forms (Fishman and Chien, 1997), and so chamber emergence takes place while cardiomyocytes are contracting and while blood is flowing. The biomechanical forces associated with contractility and blood flow have been suggested to play important roles in driving multiple aspects of cardiac morphogenesis (Bartman and Hove, 2005; Bartman et al., 2004; Hove et al., 2003). In particular, our prior work has implicated the hemodynamic forces associated with blood flow in the regulation of chamber emergence: when blood flow is reduced, cardiomyocytes fail to expand normally at the outer curvature of the embryonic zebrafish ventricle (Auman et al., 2007). This impact of blood flow on cardiomyocyte cell size suggests that hemodynamics could have a major influence on hypertrophic growth during chamber emergence; however, it is not yet known whether blood flow influences myofibril growth and organization in vivo.

Here, we use zebrafish to examine the dynamics of individual ventricular cardiomyocyte behaviors during cardiac chamber emergence. In contrast to the amniote heart, the zebrafish heart exhibits little cardiomyocyte proliferation while chambers form (Auman et al., 2007; de Pater et al., 2009; Ribeiro et al., 2007), allowing us to focus our attention on the contributions of hypertrophic growth. Taking advantage of the optical accessibility of the zebrafish heart, we have developed a new method for monitoring changes in cell surface area and myofibril content in live embryos. Our data show that hypertrophy of individual cardiomyocytes is common during ventricular chamber emergence and that hypertrophic cardiomyocytes often neighbor each other. However, to our surprise, our studies also reveal a previously unappreciated heterogeneity in ventricular cardiomyocyte behavior during this phase of development. Additionally, we find that, when blood flow is reduced, myofibril maturation and cell size expansion are dampened, the extent of cardiomyocyte hypertrophy is diminished, and coordination of neighboring hypertrophic cells is less frequent. Together, our data illuminate new aspects of the dynamic cellular mechanisms underlying cardiac chamber emergence and highlight the multiple influences of blood flow on the regulation of this process.

Materials and methods

Zebrafish

In addition to wild-type zebrafish embryos, we employed embryos homozygous for the previously described zebrafish mutation *weak atrium*^{m58} (*wea*) (Stainier et al., 1996), which disrupts the *atrial myosin heavy chain* (*amhc*) gene (Berdougo et al., 2003). When necessary, we used PCR genotyping to identify *wea* mutant embryos: the primer pair used (forward: 5′-TACGCGCAACAACTTGAA-3′; reverse: 5′-TTGCTTCTTGTTCCTCTTCAAATTGCTCTCTGATT-3′) generates an AseI site in the mutant fragment. For live imaging experiments, we injected 1.25 ng of a previously characterized anti-*amhc* morpholino (MO) (5′-ACTCTGCCATTAAAGCATCACCCAT-3′; Berdougo et al., 2003) into wild-type embryos at the 1-cell stage. We have previously demonstrated that this MO dose recapitulates the *wea* mutant phenotype, including its noncontractile atrium, reduced blood flow, and dysmorphic ventricle (Berdougo et al., 2003).

Transgenes

To achieve fluorescent labeling of cell membranes, we employed the *mkate* red fluorescent protein gene (Shcherbo et al., 2009) tagged with a CAAX membrane-targeting motif at its 3′ end. To achieve fluorescent labeling of Z-bands, we employed the zebrafish *α-actinin3b* (*actn3b*) gene tagged with *egfp* at its 3′ end. This strategy has been used previously to label Z-bands in cultured cardiomyocytes and in zebrafish skeletal and cardiac muscle (Dabiri et al., 1997; Wang et al., 2011; Zhang et al., 2009). The zebrafish *actn3b* isoform of *α-actinin* is expressed in skeletal muscle (Holterhoff et al., 2009). Actn3b shares 82% amino acid identity with Actn2, an α-Actinin isoform found in the zebrafish heart (Holterhoff et al., 2009). We chose to incorporate *actn3b* into our transgene because it was the only zebrafish *α-actinin* gene that had been clearly annotated when we initiated this work.

To drive expression of fusion proteins specifically in cardiomyocytes, genes were placed downstream of the *myl7* promoter (Huang et al., 2003). Stably integrated transgenic lines for *Tg*(*myl7:actn3b-egfp*) and *Tg*(*myl7:mkate-caax*) were created using established techniques for Tol2 transposon-based transgenesis (Fisher et al., 2006). We bred adults carrying a single allele of *Tg*(*myl7:actn3b-egfp*) to adults carrying multiple different alleles of *Tg*(*myl7:mkate-caax*) in order to generate embryos carrying both transgenes. Neither transgene causes any observable abnormalities in cardiac development or function.

Immunofluorescence

Embryos were incubated in muscle relaxation buffer (20 mM imadazole, 5 mM EGTA, 7 mM $MgCl_2$, 5 mM creatine phosphate, 10 mM ATP, 100 mM KCl) for 1.5 h prior to fixation in 4% paraformaldehyde in PBS. This relaxation buffer has been previously employed for an independent analysis of cardiac myofibrils in zebrafish (Huang et al., 2009). To visualize Z-bands and cell outlines simultaneously, we used a monoclonal anti-α-actinin antibody (Sigma, clone EA53) at 1:1000 in conjunction with rhodamine-conjugated phalloidin (Molecular Probes) at 1:50. Alternatively, we used an anti-Dm-grasp antibody (ZIRC, zn−5) at 1:100 to label cell boundaries in *Tg*(*myl7:actn3b-egfp*) embryos. Secondary antibodies used were Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, A11001) at 1:200 to recognize the anti-α-actinin antibody and Rhodamine Red-X goat anti-mouse IgG (Invitrogen, R6393) at 1:200 to recognize the anti-Dm-Grasp antibody. Images were acquired either with a 25× dry objective on a Zeiss 510 confocal microscope or a 20× dry objective on a Leica SP5 confocal microscope. Image stacks were generally composed of 15 to 20 optical slices that were 1 micron thick.

For the data set depicted in Fig. 3, three wild-type ventricles were analyzed at each timepoint. A total of 25, 35, and 62 cells were analyzed at 24, 36, and 48 h post-fertilization (hpf), respectively. For the data set depicted in Fig. 6, five or six ventricles of each genotype were analyzed at each timepoint. From wild-type ventricles, a total of 17, 74, and 50 cells were analyzed at 28, 38, and 50 hpf, respectively. From *wea* mutant ventricles, a total of 21, 62, and 64 cells were analyzed at 28, 38, and 50 hpf, respectively.

Live imaging

Embryos carrying the transgenes *Tg*(*myl7:mkate-caax*) and *Tg*(*myl7:actn3b-egfp*) were anesthetized with tricaine and mounted laterally with the right side, and therefore the ventricle, positioned toward the objective. Images were acquired with a 20× dry objective on a Leica SP5 confocal microscope. Image stacks were generally composed of 15 to 20 optical slices that were 1 micron thick. After image acquisition at the first timepoint, embryos were revived from anesthesia and incubated at 28 °C until the second timepoint, when the imaging procedure was repeated. We chose to acquire images at 40 and 45 hpf because these stages offered easy optical access to the ventricular outer curvature, which tends to be blocked by the head at earlier stages and is often obscured by the hatching gland at later stages. Cells from the central portion of the imaged area were chosen for analysis when they were crisply rendered in the xy plane of