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Mef2cb regulates late myocardial cell addition from a second heart field-like population of progenitors in zebrafish

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ABSTRACT

Two populations of cells, termed the first and second heart field, drive heart growth during chick and mouse development. The zebrafish has become a powerful model for vertebrate heart development, partly due to the evolutionary conservation of developmental pathways in this process. Here we provide evidence that the zebrafish possesses a conserved homolog to the murine second heart field. We developed a photoconversion assay to observe and quantify the dynamic late addition of myocardial cells to the zebrafish arterial pole. We define an extra-cardiac region immediately posterior to the arterial pole, which we term the late ventricular region. The late ventricular region has cardiogenic properties, expressing myocardial markers such as *vmhc* and *nkx2.5*, but does not express a full complement of differentiated cardiomyocyte markers, lacking *myl7* expression. We show that *mef2cb*, a zebrafish homolog of the mouse second heart field marker *Mef2c*, is expressed in the late ventricular region, and is necessary for late myocardial addition to the arterial pole. FGF signaling after heart cone formation is necessary for *mef2cb* expression, the establishment of the late ventricular region, and late myocardial addition to the arterial pole. Our study demonstrates that zebrafish heart growth shows more similarities to murine heart growth than previously thought. Further, as congenital heart disease is often associated with defects in second heart field development, the embryological and genetic advantages of the zebrafish model can be applied to study the vertebrate second heart field.

Introduction

The heart is the first functional organ to form in the vertebrate embryo. Bilateral populations of cardiac progenitors fuse at the midline to form the linear heart tube, which later loops to form the two-, three-, or four-chambered heart (Srivastava, 2006). Growth and morphogenesis proceed while the heart tube beats, putting novel developmental constraints on cell migration, proliferation, and differentiation. As heart growth has been primarily studied in terrestrial vertebrates, such as the mouse and chick, the evolutionary conservation of heart growth has not been closely studied.

The study of cardiac progenitors in vertebrate heart development was greatly advanced by the discovery of *Nkx2.5*, a homologue of the *Drosophila tinman* gene, whose mutation leads to loss of the fly heart equivalent, the dorsal vessel (Bodmer, 1993). Vertebrate *Nkx2.5* expression marks the bilateral cardiac progenitors (Chen and Fishman, 1996; Lints et al., 1993), and it was at first assumed that these initial *Nkx2.5*-positive cells represented all progenitors of the future myocardium. The more recent discovery of the second heart field (SHF) in chick (Mjaatvedt et al., 2001; Waldo et al., 2001) and mouse (Buckingham et al., 2005; Kelly et al., 2001) has highlighted the complexity of early heart development. At the early cardiac crescent stage, the first heart field (FHF) is found lateral and ventral to the SHF and expresses heart differentiation markers before the SHF (Cai et al., 2003). SHF cells, found in splanchnic mesoderm located dorsal to the midline heart, are a population of cardiac progenitors that migrate into the heart tube after it has been formed. Lineage tracing experiments have suggested that discrete populations of heart progenitors contribute to restricted regions of the heart (de la Cruz et al., 1977; Meilhac et al., 2004). The SHF forms the majority of the right ventricle and the entirety of the inflow and outflow tracts of the heart. The FHF forms the initial linear heart tube and forms the entire left ventricle. Both the FHF and SHF contribute cells to the atria. FGF, Wnt, BMP, and Hh signaling have been shown to affect the proliferation, differentiation, migration, and survival of SHF cells (Rochais et al., 2009). SHF regulators, such as *Isl1* (Cai et al., 2003) and *Mef2c*, have been identified. The right ventricle and outflow tract are missing in *Isl1* mutants, which is phenocopied by deletion of *Mef2c* (Lin et al., 1997), a direct transcriptional target of *Isl1* (Dodou et al., 2004). Deficiencies in SHF contribution during heart development have been shown to cause congenital heart defect (CHD)-like phenotypes in mouse models, including mispatterning of the outflow tract and septal defects (Dyer and Kirby, 2009; Horsthuis et al., 2009). As

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CHDs affect up to 1% of live births, a greater understanding of how cells of the SHF are recruited to and differentiate in the heart is essential.

The early embryonic origins of the FHF and SHF are still poorly understood (Meilhac et al., 2004). Further, the evolutionary origin of the SHF is unclear. *Isl1* is expressed in *Xenopus laevis* (Brade et al., 2007) and *Drosophila* (Mann et al., 2009) embryos and is required for heart and dorsal vessel development, respectively. In the zebrafish, recent work has shown that formation of the heart tube is followed by later phases of cardiomyocyte differentiation, with *isl1* and FGF signaling regulating addition to the venous and arterial poles of the heart, respectively (de Pater et al., 2009). It remains an open question if an SHF-like population is conserved in the simpler two-chambered heart of zebrafish. As defects in the SHF are key to many CHDs, this is a critical issue in considering the use of zebrafish to study these diseases. The conservation of mouse SHF markers and regulators in zebrafish heart development would constitute strong evidence for a more ancient origin of the vertebrate SHF.

In this study, we employed a photoconvertible fluorescent protein to study the dynamic addition of cardiomyocytes to the arterial pole of the zebrafish heart. As previously reported (de Pater et al., 2009), we observed early and late phases of myocardial cell addition. To further characterize late myocardial cell addition, we measured cell addition between 24 hours post-fertilization (hpf) and 72 hpf. The majority of addition to the arterial pole occurs prior to 36 hpf, with low addition occurring up to 2.5 days post-fertilization (dpf). We show that the cells contributing to the early heart tube and the later addition to the arterial pole are derived from the same pre-gastrula region of the embryo, suggesting a shared progenitor. We discover the existence of a cardiogenic region posterior and adjacent to the arterial pole, which we term the late ventricular region. We identify *mef2cb* as a novel regulator of zebrafish early and late heart development that is expressed in the late ventricular region and show that it is necessary for late myocardial addition. We show that FGF signaling after 20 hpf is required for the establishment of the late ventricular region and the late myocardial addition. Our data provide strong evidence that the late cell addition to the arterial pole of the zebrafish heart is analogous to the mouse SHF, in particular the *Mef2c*-dependent anterior heart field population. The ability to monitor with relative ease the dynamics of heart cell addition in the zebrafish therefore represents a novel method to study the regulation and development of the vertebrate SHF.

Materials and methods

Transgenic zebrafish lines

An *nlsKikGR* cassette was made by subcloning the *KikGR* coding sequence (Tsutsui et al., 2005) downstream of a nuclear localization signal. Zebrafish *myl7* (900 bp) and *kdrl* (7 kb) promoter elements (Beis et al., 2005; Huang et al., 2003) were subcloned upstream of an *nlsKikGR* cassette in between the minimal Tol2 transposon arms (Urasaki et al., 2006) in a pBluescript backbone vector. Stable *Tg(myl7:nlsKikGR)*^{hsc6} and *Tg(kdrl:nlsKikGR)*^{hsc7} zebrafish lines were made using standard Tol2 transgenesis approaches (Kawakami, 2005). The *myl7:EGFP*^{twu34} line used has been previously described (Huang et al., 2003).

Embryo maintenance, transplantation and microinjection

Zebrafish embryos were grown at 28 °C in embryo medium as previously described (Westerfield, 1993). Standard techniques were used for transplantation and microinjection approaches. For rescue experiments, morpholino-resistant *mef2cb* mRNA was created using the mMESSAGE mMACHINE kit (Applied Biosystems). Mutant *mef2cb* mRNA bearing five silent substitutions in the morpholino-binding sequence (5′-ATGGG**CC**G**G**AA**G**AA**A**ATTCAGATCAC-3′, where bold letters denote mutated positions) was subcloned into pCS2+ for in vitro transcription of mRNA. mRNA (20 pg) per embryo was injected for rescue. *nlsKikGR* mRNA was created using the mMESSAGE mMACHINE kit (Applied Biosystems). Transplantation was performed using 1) *myl7:nlsKikGR* transgenic donor embryos and wildtype host embryos and 2) *myl7:EGFP* + tetramethylrhodamine dextran (10,000 MW, Invitrogen) transgenic donor embryos and wildtype host embryos. At sphere stage (4 hpf), 10–20 cells were transplanted from the donor embryo to the margin of host embryos. For *myl7:nlsKikGR* transplants, UV illumination of transplant embryos was performed at 24 hpf and embryos were imaged at 48 hpf.

Photoconversion and image analysis

Photoconversion was carried out on a Zeiss Lumar V12 stereomicroscope using fluorescent light passed through the DAPI filter (Zeiss, 485049) until all green fluorescence was lost in the heart (approximately 1–2 min). Subsequent imaging of the heart was performed on a Zeiss Axiovert 200M Spinning Disk Confocal. To mount the embryos for imaging, embryos were fixed in 4% PFA for 5 min and washed three times in PBS. Two to five embryos were placed on a glass slide and covered gently by a cover slip. Image analysis was performed using the Velocity 5 Software Suite (Improvision). Ventricular cells were counted using the Point Tool. The green channel was turned off periodically to ensure that cells counted did not contain low levels of red fluorescence.

Localized photoconversion

Embryos were injected with 100 pg of *nlsKikGR* mRNA at the one-cell stage. At 24 hpf, localized photoconversion was performed on the Zeiss Axio Imager M1 microscope, using the fluorescence diaphragm to restrict the area of photoconversion to the distal tail. Each embryo was grown in the dark and imaged under identical exposure and gain settings at 24, 48, and 72 hpf on a Leica M205 FA microscope.

Morpholinos

Morpholinos were purchased from Gene Tools (Oregon, USA). The *mef2cb* morpholino was designed against the ATG site of *mef2cb* to block translation (5′-CTGAATCTTTTTTCTCCCCATTGTC-3′, the translational start site is underlined). Injection of 0.5 ng of morpholino at the one-cell stage yielded a heart-specific phenotype.

Chemical inhibition of FGF signaling

SU5402 was purchased from Tocris Biosciences (Missouri, USA). Embryos were treated either with 10 μM SU5402 in 1% DMSO or in 1% DMSO alone from 20 to 30 hpf. Inhibitor was removed by three washes with fresh embryo medium.

RNA in situ hybridization

RNA in situ hybridization was performed as previously described (Thisse and Thisse, 2008) using riboprobes specific for *myl7*/*cmlc2*, *vmhc*, *amhc*, and *nkx2.5* (Chen and Fishman, 1996; Yelon et al., 1999). For *mef2cb* riboprobe, 800 bp of *mef2cb* sequence was amplified (se primer 5′-CACGGATTATGGATGAACGCAACAGA-3′ and as primer 5′-CCAGTGATTGCGCAGACTGAGAGTTG-3′) and cloned into the pGEM-T Easy Vector (Promega). Plasmids were linearized and DIG-labeled probes made using a DIG RNA Labeling Kit (Roche). In situ images were captured on a Leica M205 FA microscope.

Bulbus arteriosus staining

The bulbus arteriosus was visualized using the NO indicator DAF-2DA as previously described (Grimes et al., 2006). Live embryos were