Utilizing Zebrafish to Understand Second Heart Field Development

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Abstract

Heart formation relies on two sources of cardiomyocytes: the first heart field (FHF), which gives rise to the linear heart tube, and the second heart field (SHF), which gives rise to the right ventricle, the outflow tract, parts of the atria, and the inflow tract. The development of the SHF is of particular importance due to its relevance to common congenital heart defects. However, it remains unclear how the SHF is maintained in a progenitor state while the FHF differentiates. Likewise, the factors that trigger SHF differentiation into specific cardiac cell types are poorly understood. Investigation of SHF development can benefit from the utilization of multiple model organisms. Here, we review the experiments that have identified the SHF in zebrafish and investigated its contribution to the poles of the zebrafish heart. Already, zebrafish research has illuminated novel positive and negative regulators of SHF development, cementing the utility of zebrafish in this context.

Keywords

Second heart field • Zebrafish • Outflow tract • Inflow tract

25.1 Introduction

The embryonic origins of the heart have been a topic of intense interest due to the prevalence of congenital heart defects [1]. Cardiac progenitors (CPs) from the first heart field (FHF) form the initial heart tube, and CPs from the second heart field (SHF) contribute to most of the structures of the mature heart including the outflow tract, right ventricle, and much of the atria [2]. The SHF is generally defined as a

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population of CPs that originates adjacent to the FHF, differentiates after the initial heart tube has formed, and is responsible for cardiomyocyte accretion at both poles of the heart tube [2]. The SHF is particularly significant to congenital heart disease; many common cardiac abnormalities are caused by defects in SHF-derived tissues, including ventricular and atrial septal defects, transposition of the great arteries, and double outlet right ventricle [3]. Despite the importance of the SHF, the mechanisms that distinguish FHF and SHF development remain unclear. What signals or factors prevent the SHF from differentiating while the FHF is deployed, and what eventual change triggers SHF differentiation? Recent advances in zebrafish research offer new approaches that can complement work in mice to deepen our comprehension of SHF regulation.

Several lines of evidence indicate the presence of a population of late-differentiating CPs in zebrafish that is likely to be analogous to the mammalian SHF. The conservation of the SHF provides exciting opportunities to advance our understanding using the distinct advantages of zebrafish embryos [4]. Zebrafish embryos develop rapidly and have small hearts that are particularly tractable for cellular resolution of cardiogenesis. Furthermore, the transparency of the zebrafish embryo facilitates exceptional opportunities for time-lapse imaging of heart formation and tracking of cardiac cell fates. Finally, zebrafish are particularly well suited for conducting both genetic and chemical screens, which have the potential to identify novel regulators of heart development. Here, we review the studies that support the existence of a zebrafish SHF and demonstrate the utility of the zebrafish for opening new avenues in SHF research.

25.2 Late-Differentiating Cardiomyocytes Originate from the SHF in Zebrafish

Two types of assays have demonstrated that late-differentiating cardiomyocytes are recruited to the poles of the zebrafish heart tube. First, a developmental timing assay that relies on the different kinetics of GFP and DsRed fluorescence was used to visualize the dynamics of cardiomyocyte differentiation. Analysis of *Tg(myl7:GFP); Tg(myl7:DsRed)* embryos showed that newly differentiated cardiomyocytes populate the cardiac poles at 48 h postfertilization (hpf), whereas cardiomyocytes in the middle of the heart differentiate at an earlier stage (Fig. 25.1a; [5]). Second, photoconversion assays have consistently revealed late-differentiating cardiomyocytes in the outflow tract. UV exposure of *Tg(myl7:kaede)* or *Tg(myl7:KikGR)* embryos after the heart tube has formed, followed by imaging at 48 hpf, showed addition of cardiomyocytes to the outflow tract after the time of photoconversion (Fig. 25.1b, [5, 6]). Together, these experiments revealed the existence of late-differentiating cardiomyocytes at the arterial pole of the zebrafish heart that seem to be analogous to SHF-derived cardiomyocytes in mammals.

Fate mapping in zebrafish has shown that early SHF precursors seem to neighbor the FHF. Prior to gastrulation, arterial pole progenitors are found adjacent to ventricular progenitors at the embryonic margin (Fig. 25.1c; [7]). After

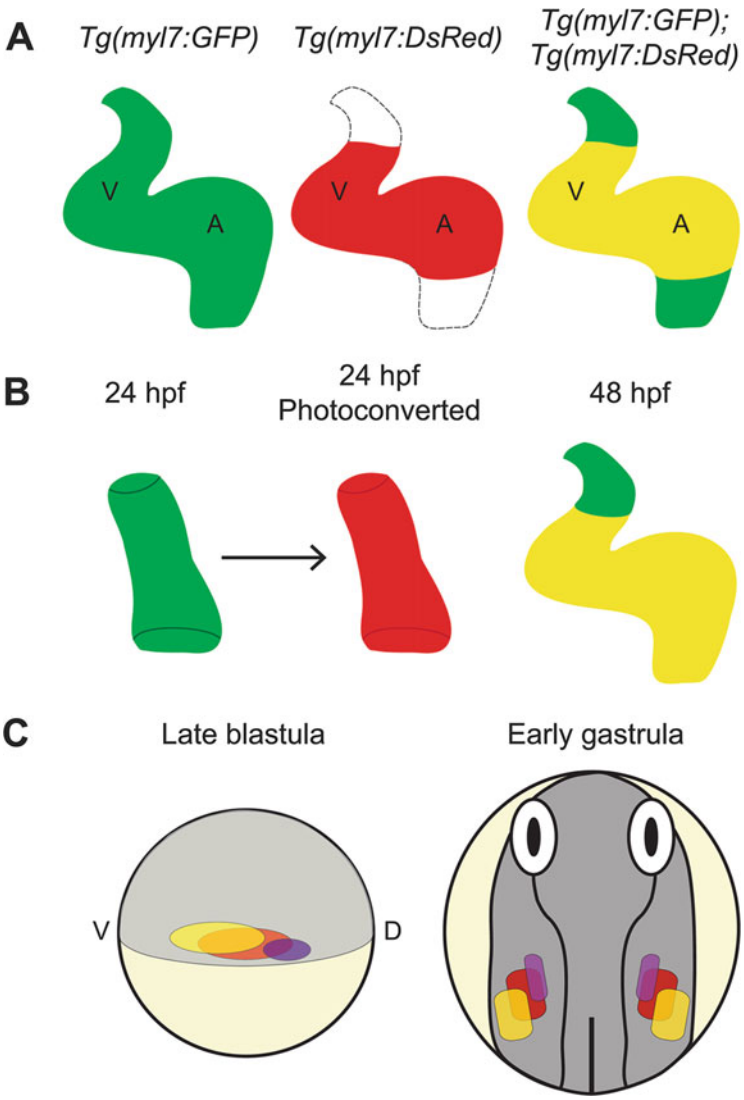

Fig. 25.1 Late-differentiating cardiomyocytes originate from the zebrafish SHF. (**a**) A developmental timing assay reveals late-differentiating cardiomyocytes displaying GFP, but not DsRed [5]. (**b**) Green-to-red conversion of photoconvertible proteins expressed in differentiated cardiomyocytes at 24 hpf, followed by imaging at 48 hpf, reveals newly added green cardiomyocytes in the outflow tract [6]. (**c**) Fate mapping in the late blastula shows that outflow tract progenitors (*purple*) are located close to the margin, adjacent to ventricular progenitors (*red*), and separate from atrial progenitors (*yellow*) [7]. In the early gastrula, outflow tract progenitors are located in a medial cranial portion of the ALPM [7]

gastrulation, arterial pole progenitors map to a medial cranial region next to the FHF in the anterior lateral plate mesoderm (ALPM) (Fig. 25.1c; [7]). Finally, DiI labeling has shown that the SHF resides adjacent to the heart tube in older embryos: pericardial cells just outside the outflow tract at 24 hpf move into the arterial pole at later stages [7]. The SHF has also been identified using Cre-mediated lineage tracing. This technique has shown that arterial pole progenitors express both *gata4* and *nkx2.5* during somitogenesis, confirming that SHF progenitors originate in the ALPM [8]. Furthermore, Cre-mediated lineage tracing has confirmed that cells from the pericardial mesenchyme adjacent to the heart tube migrate into the outflow tract [9]. Taken together, these analyses show that the late-differentiating cardiomyocytes at the zebrafish arterial pole meet the criteria that define the SHF. Outflow tract cells remain undifferentiated until after the linear heart tube has formed, are recruited to the arterial pole from outside the heart, and map to an area adjacent to the FHF. These data, combined with conserved molecular mechanisms regulating mouse and zebrafish arterial pole development, suggest that the SHF is a conserved vertebrate feature.

25.3 Mechanisms Regulating Outflow Tract Development in Zebrafish

Studies of the regulation of outflow tract formation have demonstrated conservation of the transcription factors utilized in zebrafish and mice. Zebrafish embryos deficient in *mef2cb* lack late-differentiating cells that form the outflow tract [6], which is strikingly similar to the phenotype of *Mef2c* mutant mice that lack the SHF-derived outflow tract and right ventricle [10]. Zebrafish *tbx1* mutants have several outflow tract defects, including reduced migration of cells into the heart [7] and reduced proliferation of cells at the arterial pole, resulting in a small outflow tract [11]. This phenotype is reminiscent of mouse *Tbx1* mutants, which also display outflow tract abnormalities due to severely reduced proliferation in the SHF [12].

Signaling pathways also seem to have conserved roles in the mouse and zebrafish SHF. Hedgehog signaling is important for zebrafish SHF development; migration of cells into the heart is impaired in *smoothened* mutants, resulting in a small outflow tract [7]. Similarly, hedgehog signaling is crucial for mammalian SHF survival and outflow tract septation [13]. In zebrafish, reduced FGF signaling eliminates accretion of cardiomyocytes at the arterial pole [5] and blocks *mef2cb* expression in the SHF [6]. This requirement for FGF signaling mimics mouse *Fgf8* mutants, which have a severely hypoplastic outflow tract and right ventricle [13]. These findings underscore the conserved mechanisms regulating outflow tract development and suggest that new discoveries in the zebrafish SHF are likely to be relevant to mammals.

Importantly, novel insights into outflow tract development have emerged through studies in zebrafish. The role of Ltbp3, a secreted protein that regulates TGF-β ligand availability, has been of particular interest. *ltbp3* is expressed in the

zebrafish SHF, and Cre-mediated lineage tracing has shown that *ltbp3*-expressing cells give rise to outflow tract cardiomyocytes [9]. Ltbp3-deficient embryos lack an outflow tract due to reduced SHF proliferation, a consequence of reduced TGF-β signaling [9]. This work not only illuminated Ltbp3 as a new SHF regulator but also uncovered a novel role for TGF-β signaling in SHF development. Additional studies have revealed that Nkx2.5 promotes maintenance of *ltbp3* expression [8]. This is exciting, as it elucidates a new pathway downstream of Nkx2.5: Nkx2.5 facilitates the activation of TGF-β signaling through regulation of *ltbp3* and thereby drives SHF proliferation. Since Nkx2.5 is highly relevant to congenital heart disease, factors downstream of Nkx2.5 are excellent candidates for translational research. Thus, investigations in zebrafish can lead to the discovery of novel regulators of SHF development and provide new insight into connections between important factors.

25.4 Mechanisms Regulating Inflow Tract Development in Zebrafish

In mice, the SHF has been shown to contribute to the venous pole in addition to the arterial pole [2]. The mammalian SHF is thought to be subdivided into the anterior SHF, which gives rise to the right ventricle and outflow tract, and the posterior SHF, which gives rise to the atria and the inflow tract [2]. The zebrafish heart has a distinct population of inflow tract cells that express the canonical SHF marker Isl1 [14]. In addition, developmental timing assays have shown that the zebrafish inflow tract contains a population of late-differentiating cardiomyocytes (Fig. 25.1a; [5]). However, the degree of overlap between these two populations has not been examined, and the precise timing of when inflow tract cells are added to the heart is unclear. Furthermore, it is not known where zebrafish inflow tract cells originate in the early embryo and if inflow and outflow tract progenitors share a common lineage. Future experiments will be valuable to elucidate the zebrafish equivalent of the mammalian posterior SHF.

Studies of inflow tract development in zebrafish have revolved around the role of Isl1. Zebrafish Isl1 mutants lack late-differentiating cardiomyocytes at the venous pole [5]. This phenotype is similar to that of Isl1 null mouse embryos, which lack SHF-derived atrial cardiomyocytes [15]. Interestingly, studies in zebrafish have identified a novel requirement for the LIM domain protein Ajuba, which directly interacts with Isl1 [14]. Ajuba-deficient embryos have large hearts with an excess of Isl1-expressing cells and an expansion of SHF markers in the ALPM. Conversely, Ajuba overexpression eliminates Isl1 in the inflow tract [14]. Ajuba is one of the first factors that has been shown to limit SHF development, and the presence of Ajuba may determine whether Isl1 activity promotes or limits cardiomyocyte formation. The identification of Ajuba as a negative regulator of inflow tract formation further illustrates the utility of zebrafish for the discovery of novel factors involved in SHF development.

25.5 Future Directions and Clinical Implications

Altogether, the studies summarized here support the value of the zebrafish for the investigation of SHF development. It will be particularly exciting for future work in zebrafish to probe important open questions in this area. For example, zebrafish studies may be valuable for elucidating the mechanisms that pattern the SHF into its anterior and posterior subdivisions. In addition, it will be interesting to use zebrafish to examine the factors that control differentiation of multipotent SHF cells into myocardial, endocardial, and smooth muscle lineages [9]. Zebrafish will also be valuable for exploring whether multipotent SHF cells are maintained after embryogenesis, perhaps to be deployed after injury. In the long term, use of the zebrafish for analysis of SHF development is likely to illuminate pathways that facilitate our understanding of the etiology of congenital heart disease.

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