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Zebrafish in the Study of Early Cardiac Development

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Abstract

Heart development is a complex process that involves cell specification and differentiation, as well as elaborate tissue morphogenesis and remodeling, to generate a functional organ. The zebrafish has emerged as a powerful model system to unravel the basic genetic, molecular and cellular mechanisms of cardiac development and function. Here we summarize and discuss recent discoveries on early cardiac specification and the identification of the second heart field in zebrafish. In addition to the inductive signals regulating cardiac specification, these studies have shown that heart development also requires a repressive mechanism imposed by retinoic acid signaling to select cardiac progenitors from a multipotent population. Another recent advance in the study of early zebrafish cardiac development is the identification of the second heart field (SHF). These studies suggest that the molecular mechanisms that regulate SHF development are conserved between zebrafish and other vertebrates including mammals, and provide insight into the evolution of the SHF and its derivatives.

Keywords

retinoid acid signaling; Fgf signaling; second heart field; Ltbp3; zebrafish

Introduction and advantages of the zebrafish system

Cardiac diseases remain a leading cause of morbidity and mortality, and many of these diseases arise at least in part from genetic defects that impact the development and maturation of the heart. Therefore, understanding the genetic, molecular and cellular mechanisms that govern the formation, differentiation, and growth of the heart will greatly enhance our understanding of heart disease, and facilitate the identification of new approaches to repair or regenerate damaged heart muscle^{1–4}.

With the development of various genetic and cell biological tools including forward genetic screens, transgenesis, lineage tracing and cell transplantation, the zebrafish has emerged as a powerful vertebrate model organism to investigate a number of biological processes. In addition, the zebrafish offers several distinct advantages as a model to study cardiac development. First, the external development of the embryos allows a direct noninvasive

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observation of the development of the heart as it happens and at cellular resolution⁵. Second, zebrafish embryos do not initially rely on their cardiovascular system for oxygen needs. Therefore, zebrafish cardiac mutants can survive and continue to develop for several days, allowing a detailed analysis of their phenotype. Although the zebrafish heart is simpler in structure than its mammalian counterpart, genes responsible for essential steps of cardiac development are conserved throughout vertebrates. Thus, studies of cardiac development using the zebrafish as a model organism will continue to help address key questions in cardiac development. This review is not intended to cover every aspect of cardiac development in zebrafish, but rather focuses on cardiac progenitor specification as well as the recent discovery of the second heart field in zebrafish.

Early cardiac specification in zebrafish

A fundamental question in organogenesis concerns the means by which the organ progenitor cells are specified. Previous studies in *Xenopus*, chick and mouse have implicated several signaling molecules, such as Bmp, Activin/Nodal, Fgfs and Wnts as positive regulators of this process⁶⁻¹⁰. Although it is conceivable that the combinatorial action of these signaling molecules could delineate the correct number of cardiac progenitors, it was not known whether a repressive mechanism was also involved. Using a combination of genetic, pharmacological and fate mapping approaches, Keegan et al., showed that in zebrafish the potential of naïve cells to adopt a cardiogenic fate is restricted by retinoid acid (RA) signaling¹¹. *aldh1a2* (aka *neckless*) mutants, which are defective in RA synthesis, generate an excess number of cardiomyocytes. A similar cardiomyocyte expansion phenotype is also observed in embryos treated with the pan-RA receptor antagonist BMS189453 before and during gastrulation. To define the role of RA signaling in cardiac specification more precisely, the authors generated a high-resolution fate map of the lateral marginal zone of the late blastula^{11, 12}, the region where the cardiac progenitors are located¹³. They showed that twice as many cells from this region of the embryo become cardiomyocytes in BMS189453-treated animals than in controls, suggesting that the generation of extra cardiomyocytes in RA-deficient embryos results from fate transformation rather than increased cardiomyocyte proliferation. A closer examination of the role of RA signaling in cardiac specification revealed that the number of cells in both the atrial and ventricular chambers was significantly increased upon BMS treatment during gastrulation¹⁴. Additional fate mapping experiments showed that the increase in the number of ventricular cardiomyocytes was due to an increased number of ventricular progenitors. In contrast, BMS treatment did not generate more atrial progenitors but rather allowed each atrial progenitor to produce more progeny. RA signaling also appears to restrict atrial cell numbers over a longer time window. BMS treatment around 6–8 somite stage still increased the number of atrial but not ventricular cells. This restriction of atrial cells by RA signaling at early somite stages appears to be mediated by *hoxb5b*, a *hox* gene whose expression in the adjacent forelimb field is induced by RA signaling¹⁴. In *hoxb5b* morphants, the atrium is enlarged and the number of atrial, but not ventricular, cells is increased¹⁴. Thus, Hoxb5b appears to act in a cell non-autonomous fashion to restrict atrial but not ventricular fate, although the exact nature of the forelimb-derived Hoxb5b-dependent signal(s) remains to be identified. Given this specific effect of Hoxb5b on atrial cell fate, it may also be necessary to invoke an independent factor(s) that mediates the repressive effect of RA signaling on ventricular cell fate. In this regard, future studies designed to identify this factor(s) will certainly provide a more comprehensive picture of how RA signaling exerts its inhibitory effect on cardiomyocyte fate. Besides Hox5b, Fgf (*Fibroblast growth factor*) signaling also appears to act downstream of RA signaling to regulate both heart and forelimb formation¹⁵. Enhancing Fgf signaling by global overexpression of a constitutive version of Fgfr1 at the end of gastrulation phenocopied the loss of RA signaling. More importantly, both the cardiac and forelimb defects caused by RA deficiency could be rescued by morpholino

knockdown of *fgf8*, suggesting that RA signaling acts to temper Fgf signaling to ensure the proper development of both the heart and forelimb. In line with these findings in zebrafish, previous studies in mouse have revealed a similar role for RA signaling in reducing Fgf signaling to restrict the specification of cardiac progenitors^{16, 17}.

Second heart field in zebrafish

A recent advance in cardiac biology has come from the identification of two sources of cardiac progenitors in chick and mouse embryos. The first heart field (FHF) contributes mainly to the linear heart tube. Cells from the second heart field (SHF) are then recruited to the heart tube, contributing to its further growth and elaboration into a functional pumping organ¹⁸. The discovery of the SHF dates back to 1977 when de la Cruz showed that the formation of the cardiac outflow tract in chick embryos involved the recruitment of cells from a progenitor population located in the pharyngeal mesoderm¹⁹, which was later referred to as the anterior or secondary heart field. The identification of an analogous population in mouse was facilitated by a lacZ transgene inserted in the *Fgf10* (*Fibroblast growth factor 10*) locus^{20, 21}. Cells expressing this transgene are initially located in the pharyngeal mesoderm, and later become incorporated into the arterial pole of the heart. The SHF also contains the region immediately posterior to the *Fgf10*-LacZ expression domain²². This posterior portion of the SHF contributes to the venous pole of the heart. The entire SHF expresses the homeobox gene *Islet-1* (*Isl1*), a critical regulator of SHF development. In the absence of *Isl1* function, SHF derivatives such as the right ventricle and outflow tract fail to form²². Despite its primitive outflow tract and lack of a right ventricle, the zebrafish heart does require a SHF for the formation of its arterial pole^{23–26}. Studies of zebrafish SHF development suggest that the molecular mechanisms underlying this process are largely conserved with those in other vertebrates including mammals. These studies also identified new regulators of SHF development and provided novel insights into this process.

The ability to perform high-resolution live imaging in zebrafish allowed direct visualization of the addition of late cardiogenic cells to the growing heart tube. Taking advantage of the slower maturation and fluorescence properties of DsRed compared to eGFP^{27, 28}, de Pater et al., examined the temporal order of cardiomyocyte differentiation²³. With this assay, two cardiomyocyte populations were observed in the developing heart: the early differentiated eGFP and DsRed double positive cells and the newly differentiated eGFP positive and DsRed negative cells. The analysis of the distribution of these two populations at different time points, as well as Kaede photoconversion experiments, suggested two distinct phases of cardiomyocyte differentiation^{23, 25}. The linear heart tube was formed as the result of the first round of progressive cardiomyocyte differentiation, whereas the second round of cardiomyocyte differentiation gave rise to the myocardium of the arterial pole of the heart. At the linear heart tube stage and prior to its differentiation, this late cardiogenic population seems to be located at the extra-cardiac region that lies immediately adjacent to the arterial pole of the heart, and it appears to express the MADS box transcription factor gene *mef2cb* and activate the Fgf signaling pathway^{23, 25}.

Consistent with their expression/activation pattern and role in mouse arterial pole development, *Mef2cb* and Fgf signaling are both required for late cardiomyocyte addition to the arterial pole in zebrafish^{23, 25}. The development of the arterial pole in zebrafish also appears to be dependent on Shh signaling and the T-box transcription factor *Tbx1*, two other key regulators of mouse arterial pole development^{24, 29–31}. In the absence of Shh signaling or *Tbx1* function, the ventricle and outflow tract of the zebrafish heart become significantly reduced in size due to a reduction in the incorporation of late cardiogenic cells to the arterial pole²⁴. Thus, the analogous position of the late cardiogenic population relative to the linear heart tube, as well as the observation that the same genes regulate the addition of its derived

cells to the arterial pole, strongly support the notion that the late cardiogenic population in zebrafish shares characteristics with the SHF of other vertebrates including mammals.

Although the aforementioned studies clearly revealed the existence of a SHF in zebrafish, the extent to which it contributed to heart formation remained unresolved. For instance, a previous study had suggested that the arterial pole of the zebrafish heart gives rise to the distal part of the ventricle and the outflow tract³². In support of this idea, Dil labeling experiments showed that cells located in the dorsal pericardial wall adjacent to the arterial pole at the linear heart tube stage indeed contributed to the distal portion of the ventricular myocardium²⁴. But how much of the ventricular myocardium derived from the SHF remained unclear. A recent study has implicated the latent Tgf β binding protein 3 (Ltbp3), a regulator of Tgf β secretion and activation, in arterial pole development in zebrafish²⁶. Genetic tracing experiments revealed that the *ltbp3* expressing cells, which are originally located immediately adjacent to the linear heart tube, contribute significantly to the developing heart, making up the distal half of the ventricular myocardium as well as the myocardium of the proximal outflow tract. The *ltbp3* positive cells also give rise to the endothelial and smooth muscle cells of the outflow tract. In addition, it appears that Ltbp3 is specifically required for the formation of the cardiac structures that derive from the *ltbp3* expressing cells. Morpholino knockdown of *ltbp3* caused severe ventricular defects with a nearly fifty percent reduction of the number of ventricular cardiomyocytes and endocardial cells, whereas the development of the atrium was not affected. *ltbp3* morphants also failed to form the outflow tract smooth muscle cells. Although the molecular mechanisms by which Ltbp3 regulates SHF development remain unclear, the SHF progenitors in *ltbp3* morphants are specified properly, but they fail to proliferate or self-renew to replenish the progenitor pool as they differentiate and migrate into the heart. Consistent with a role for Ltbp3 in regulating Tgf β signaling, embryos treated with a pharmacological inhibitor of Tgf β signaling recapitulated the *ltbp3* morphant phenotypes, thereby for the first time implicating Tgf β signaling in controlling the proliferation or renewal of the SHF progenitors during arterial pole development.

Although roles for Fgf signaling, Tbx1 and Mef2c have been clearly established in zebrafish SHF development, the specific aspects of SHF development regulated by these factors remain to be determined. For instance, we need to understand whether these signaling pathways and transcription factors regulate the specification, proliferation, differentiation, or survival of SHF cells or simply the addition of the SHF cells to the heart tube. In addition, it will also be important to investigate how the different signaling pathways and transcription factors interact to coordinate the specification, proliferation, differentiation and migratory behaviors of the SHF progenitors.

The identification of a SHF in zebrafish has important implications for understanding congenital heart disease. Defects in SHF development, such as mis-specification of the SHF progenitors or the failure of the addition of SHF cells to the heart tube, often lead to congenital heart disease. As a genetically tractable system, zebrafish offers unique advantages to study SHF development as the transparency of the embryos allows a detailed study of cell behaviors in wildtype and mutant animals.

Evolutionary implications of the identification of a SHF in zebrafish

During the transition from aquatic to terrestrial life, vertebrate hearts acquired a new set of cardiac components to separate the systemic and pulmonary circulations. The newly acquired cardiac segments for pulmonary circulation, such as the right ventricle are mostly derived from the SHF^{33, 34}. Thus, the identification of a SHF in zebrafish, an organism that does not have a separate pulmonary circulation has important evolutionary implications, and argues

against the idea that the SHF and the pulmonary circulation co-evolved in terrestrial vertebrates. Interestingly, the zebrafish ventricle and the primitive ventricle of “higher” vertebrates appear to be patterned in a similar fashion. In both cases, SHF derived cells contribute to the formation of a distinct segment of the ventricle²⁶.

Although many of the molecular mechanisms that regulate the development of the SHF and its derivatives are conserved between zebrafish and higher vertebrates, it is worth noting that *Islet-1*, a key regulator of mouse SHF development, does not appear to be required for arterial pole formation in zebrafish²³. Cardiomyocyte differentiation at the venous pole, however, was affected by the loss of *Islet-1* function. In zebrafish *islet-1* mutants, *bmp4* expression at the venous pole is completely absent whereas its expression at the atrioventricular junction and outflow tract is not affected²³. While zebrafish *Islet-1* is thus more confined to regulate venous pole cardiomyocyte differentiation, mouse *Islet-1* is required for controlling the development of both arterial and venous poles²². This broader function of mouse *Islet-1* is likely due to the fact that in mouse, the arterial and venous poles are both closely apposed to *Islet-1* expressing pharyngeal mesoderm. In zebrafish, however, the *Islet-1* positive cells in the cardiac field are not in close contact with the future arterial pole²³. Thus, one could speculate that *Islet-1* expanded its expression domain and gained a new function in controlling the development of the arterial pole as the terrestrial vertebrates emerged. Alternatively, and in light of a recent study in *Ciona*³⁵, it is also possible that zebrafish lost the *islet-1* expression pattern that allows it to regulate arterial pole development.

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Non-standard Abbreviations and Acronyms

RA	Retinoid Acid
Fgf	Fibroblast growth factor
LPM	lateral plate mesoderm
SHF	second heart field
Mef2ca	Myocyte-specific enhancer factor 2C
Tbx	T-box
Ltbp3	latent Tgf β binding protein 3
hpf	hours post-fertilization

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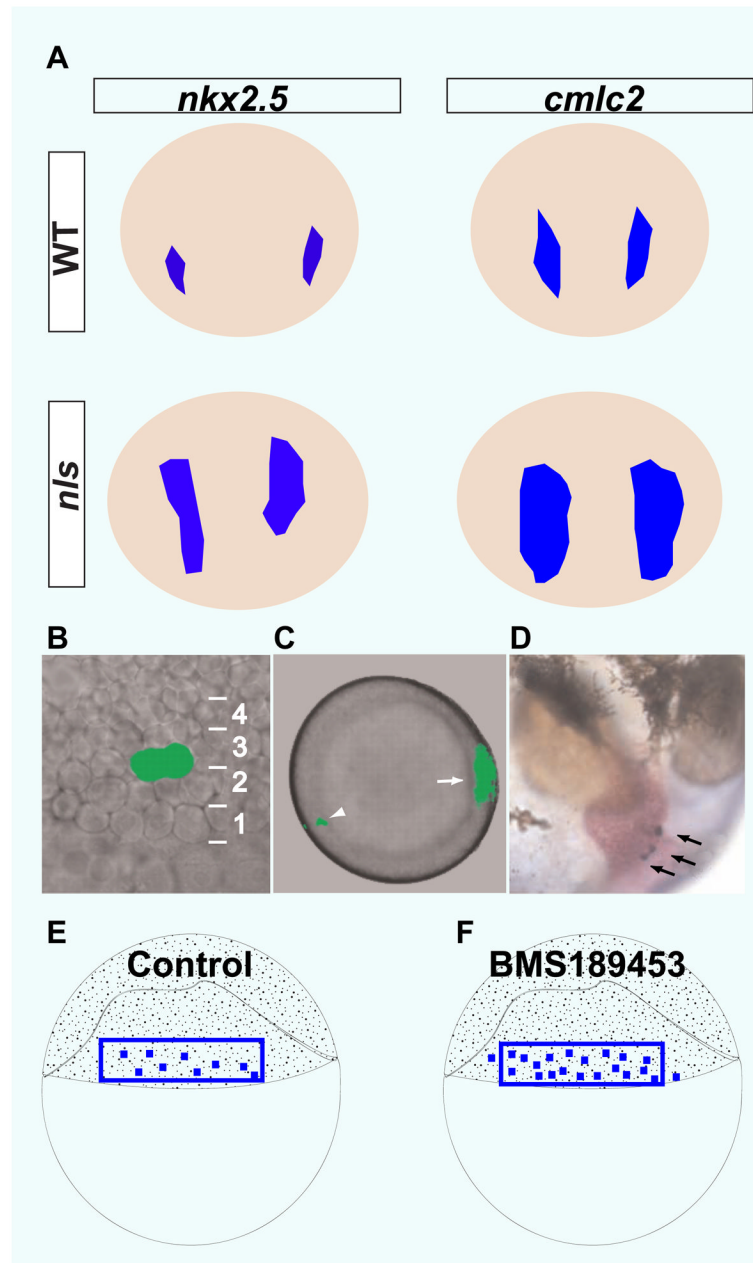


Fig 1.

RA signaling restricts cardiogenic potential. A) *nkx2.5* and *cmlc2* expression domains are expanded in *aldh1a2* (*aka neckless*) mutant embryos compared to wildtype (6-somite stage for *nkx2.5* expression and 16-somite stage for *cmlc2* expression). Dorsal views, anterior to the top. B–D) Construction of blastomere fate map with caged fluorescein. B) Lateral view of two neighboring blastomeres (40% epiboly stage) in tier 3 that were labeled by activating caged fluorescein. C) Animal pole view of labeled blastomeres (arrowhead). The dorsal midline was labeled by *Tg(gsc:GFP)* expression (arrow). D) Descendants of the labeled blastomeres were identified in the heart (arrows, 44 hpf (hours post-fertilization)). (E–F) Schematics illustrate that more blastomeres have cardiogenic potential in BMS189453-treated embryos than in control (40% epiboly blastula stage). (adapted from Keegan BR et al., *Science*. 307:207–209).

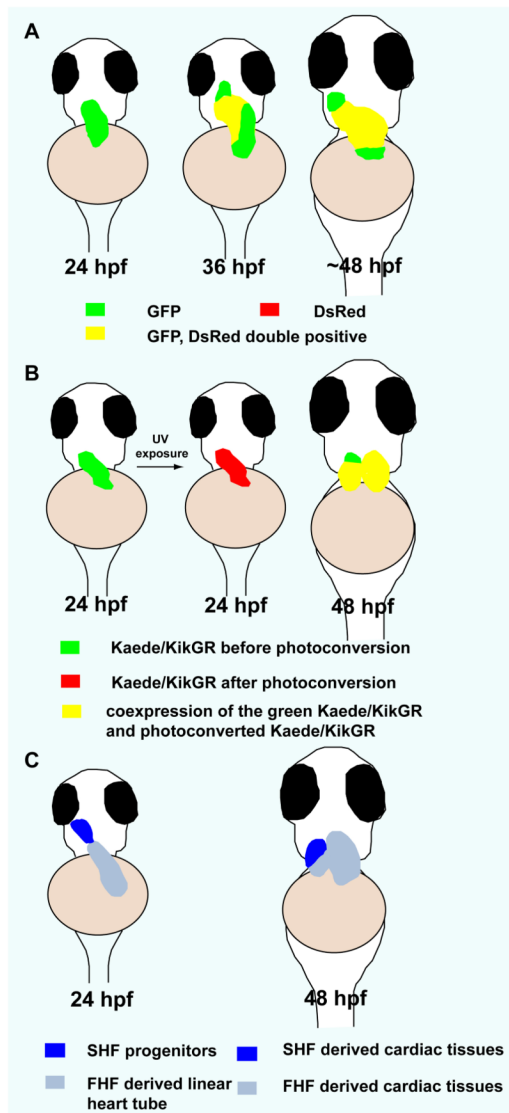


Fig 2. Second heart field in zebrafish. A) Temporal order of cardiomyocyte differentiation: newly differentiated cardiomyocytes (green), earlier differentiated cardiomyocytes (yellow). B) Kaede/kikGR photoconversion experiments suggest that cardiomyocytes at the arterial pole of the zebrafish heart differentiate after the formation of the linear heart tube (green). C) Contribution of the SHF to zebrafish heart development. At 24 hpf, SHF progenitors are located immediately adjacent to the ventricle. At 48 hpf, SHF derived cells populate the distal segment of the ventricle as well as the outflow tract. Ventral views, anterior to the top. (adapted from de Pater E et al., *Development*. 136:1633–1641, Lazic and Scott, *Developmental biology*. 354:123–133, and Zhou Y et al., *Nature*. 474:645–648)