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Zebrafish as a model for cardiovascular development and disease

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Understanding the molecular and cellular mechanisms underlying normal development and pathological conditions is an important step towards prevention and developing effective therapeutic approaches for cardiovascular diseases. In recent years, the zebrafish model has rapidly emerged as a powerful genetic system to study cardiac development and function, and some zebrafish mutants have been used as animal models to dissect the molecular causes of cardiovascular disease. Here, we discuss current advancements in zebrafish cardiovascular studies with a focus on the genetic mechanisms regulating cardiac function and the potential of utilizing zebrafish for drug discovery.

Zebrafish heart development

Although the morphological differences between the zebrafish and human heart may seem astounding, the fish heart and mammalian heart undergo similar morphogenetic processes. Cardiac cells of the zebrafish begin as two lateral populations, migrating to the midline and forming the primitive heart tube. As the embryo develops, the heart tube undergoes cardiac looping and further differentiates into distinct cardiac chambers separated by valve leaflets at the atrioventricular (AV) junction [1,2]. Recent optical imaging studies define four distinct physiological developmental stages of zebrafish conduction system that correspond to the cellular and anatomical changes of the heart [3]. Spontaneous, uncoordinated contraction of cardiomyocytes is noted soon after the fusion of bilateral cardiac precursors. By 24 hours post fertilization (hpf), the conduction system begins as a linear wave that travels through the heart from the sinus venosus to the outflow tract, coinciding with the observed peristaltic contraction pattern of the primitive heart tube. By 48 hpf, cardiomyocytes at the AV ring differentiate into cells with slow conducting properties, creating a significant conduction delay at the AV boundary and transitioning the heart from peristaltic contraction to coordinated contraction of the chambers [3,4]. As the ventricle

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continues to thicken and develop trabeculae, a fast conduction network forms within the ventricle, where a complex conduction pattern proceeding from the outer curvature of the ventricle to its base is observed. Finally, upon maturation of the fast conduction network, the conduction wave transforms to an apex-to-base model across the ventricular myocardium [3,5].

Zebrafish as a genetic model for cardiovascular research

The zebrafish model offers several advantages in examining the genetic mechanisms behind cardiovascular development and function. First, zebrafish produce a large number of easily accessible and transparent embryos, facilitating phenotypic driven genetic screens. Second, the embryonic zebrafish heart is placed at a prominent position at the ventral side of the embryo allowing visual inspection of the patterning and function of the developing heart in live embryos. Furthermore, the zebrafish embryo obtains sufficient oxygenation by passive diffusion making circulation nonessential for the first few days of development providing a unique opportunity to inspect the progression of cardiovascular phenotypes.

A large number of mutations affecting the contractility and/or rhythmicity of the developing zebrafish hearts have been identified [6,7]. Many of these mutants exhibit phenotypic characteristics resembling human heart diseases. Shown in Table 1 are selected zebrafish mutants whose molecular lesions have been determined. Many of these loci encode zebrafish homologues of genes associated with human cardiac diseases. For instance, mutations in *essential myosin light chain*, *regulatory myosin light chain*, *titin*, *cardiac troponin T* and *beta myosin heavy chain* have been associated with cardiomyopathy in humans [8–11]. The hearts of zebrafish mutants defective in these genes also show reduced contractility, myofibril disarray and dysmorphic cardiac chambers [12–15], supporting the notion that cellular and molecular mechanisms underlying the contractile machinery are conserved among vertebrates.

In addition to the contractile machinery, mechanisms regulating cardiac rhythmicity appear to be conserved from fish to mammals. As in the mammalian heart, genes regulating ion flux are required for establishing and maintaining coordinated cardiac contraction. Mutations in human *cacna1c* have been associated with Timothy Syndrome, a severe arrhythmic disorder [17]. The heart of zebrafish embryos mutated in *L-type calcium channel subunit α1C* (*cacna1c*) never initiate coordinated contraction, rather individual cardiomyocytes contract spontaneously, which is consistent with the role of L-type calcium channels in calcium influx [16]. In addition, the zebrafish *tremblor* locus encodes the zebrafish homologue of a cardiac-specific *Na/Ca exchanger 1* (*NCX1h*, also known as *slc8a1a*) gene. Loss of function of NCX1h disrupts normal calcium transients and leads to a chaotic movement of the heart resembling cardiac fibrillation in humans [18,19]. The Ca^{2+} extrusion activity of NCX1h is modulated by the cellular concentration of Na^{+} regulated by the sodium pump. Interestingly, zebrafish embryos deficient in Na,K-ATPase α1b1 also develop cardiac fibrillation [20], further indicating the critical role of calcium homeostasis in regulating cardiac rhythmicity. Furthermore, multiple mutations in the *ether-a-go-go-related* gene, KCNH2, were identified in zebrafish. Zebrafish embryos carrying loss-of-function mutations of KCNH2 have a prolonged ventricular depolarization whereas cardiac repolarization is accelerated in those embryos homozygous for an activated form of KCNH2 [21–23]. These phenotypes are consistent with the finding that loss of KCNH2 activity causes long QT syndrome, and overactivation of KCNH2 causes short QT syndrome in humans [24,25].

The conserved molecular mechanisms underlying cardiac contraction and rhythm and the phenotypic similarity between zebrafish cardiac mutants and human diseases suggest that zebrafish could serve as a genetic model for discovering genes associated with cardiac

diseases. This notion is supported by recent studies on integrin-linked kinase (ILK). ILK is a key transducer of biochemical signals initiated at the plasma membrane by cell-matrix interactions including the interaction between laminin and integrin. Zebrafish *ilk* mutant hearts have severe myocardial dysfunction resembling human dilated cardiomyopathy (DCM) [26,27]. Morpholino knockdown of *laminin α4* (*lama4*) also results in cardiac dysfunction similar to that observed in *ilk* mutants, suggesting that ILK mediated cell-matrix signaling has an essential role in regulating cardiac growth and contractility. These findings led Knoll et al. to screen patients with severe DCM and found two mutations in *LAMA4* and a mutation in the coding region of *ILK* [27]. While the causative relationship between mutations in the ILK/laminin signaling pathway and DCM warrant further investigation, these findings suggest that zebrafish may serve as a tool for discovering genetic basis of human cardiac diseases. Recently, molecular cloning of zebrafish mutants with atrioventricular block revealed the roles of transcription factors such as *foxn4* and *tcf2* in cardiac conduction [28,3]. Whether mutations in these genes also cause similar cardiac defects in mammalian hearts is currently unknown. Future studies investigating the role of genes identified from zebrafish mutants or morphants in mammals will provide further insight into the similarity or differences in mechanisms regulating cardiac function in divergent species and may help discover causes of human cardiovascular development or disease.

Chemical genetic screens in zebrafish

Genetic screens in zebrafish have generated useful resources to dissect genes and pathways critical for heart development. However, if a gene or pathway is involved in multiple biological processes, its cardiovascular role may not be easily evaluated in the mutants due to early developmental defects. One complementary approach to overcome this problem is small molecule-based chemical genetic screens. The zebrafish embryos are fertilized and developed *ex utero*. Chemical inhibitors or agonists can be applied to embryos at desired developmental stages to evaluate their effects on biological processes of interest. In fact, small molecule screens conducted in zebrafish have already discovered compounds that specifically affect the development of the heart, nervous system, hematopoietic cells and pigment cells (for review see [29]). In addition, screening compounds that suppress or enhance phenotypes in a specific mutant background may identify modifiers and/or signaling pathways critical to the biological processes of interest. This approach has been successfully applied in zebrafish. For example the *gridlock* (*grl*) mutant carries a mutation in the zebrafish *hey2* homologue and blocks aortic blood flow resembling aortic coarctation in humans [30,31]. Multiple compounds were identified in a chemical suppressor screen by their abilities to restore blood flow to the tail of *grl* mutants [32,33]. Mechanisms of action studies on these compounds lead to the discovery that *grd/hey2* regulates vessel formation through the VEGF signaling pathway and that PI3K and ERK mediating opposing effects in artery/vein specification downstream of VEGF [33]. Our laboratory also identified a novel small molecule that suppresses cardiac fibrillation in *tre/slc8a1a* mutant embryos using a similar strategy (Huang, Kwon, and Chen, unpublished data), attesting to the feasibility of this approach in zebrafish. Whether these compounds could be developed into new therapeutic agents for cardiovascular disorders remain to be seen, yet the zebrafish system affords a potent means to investigate the genetic and molecular pathways responsible for cardiac function.

The success of chemical genetic screens in zebrafish raises interests of using the zebrafish model to discover pharmaceutical agents regulating cardiac physiology. Milan et al. screened a collection of 100 biologically active small molecules for their effects on zebrafish heart rate and found 36 compounds caused bradycardia [34]. Interestingly, eighteen of these compounds were known to cause QT prolongation and torsades de pointes in humans,

supporting the feasibility of using zebrafish as a model organism to evaluate drug effects on the cardiovascular system *in vivo*. From an independent screen of 238 biologically active small molecules, we identified fifteen compounds that reduce the contractility or interfere with the rhythmic contraction of the developing zebrafish heart. Among these, seven are known modulators of ion channels or downstream signaling pathways in mammals (bepridil, amiodarone, niguldipine, pimozide, penitrem A, fluspirilene, and KN-93) [35–41] consistent with the notion that ion flux is critical in regulating cardiac function. Furthermore, these results offer additional verification that the fish heart responds to pharmacological agents in a similar manner as mammalian models.

In order to further utilize the zebrafish model to discover pharmaceutical targets regulating cardiac physiology, easily accessible, high-throughput quantitative tools are required to characterize cardiac abnormalities at functional and physiological levels. Over the years, many programs designed for quantifying cardiac contraction have been developed [42–49], including a recent effort to monitor heart rate using an automated system [50]. In addition, we developed a line-scan based methodology allowing a low-cost, high-throughput quantitative means to measure cardiac function parameters [51]. Hereafter, we will refer to this system as zCAS for zebrafish cardiac analysis system. Similar to 2D-echocardiography performed on patients, zCAS produces two-dimensional images of each cardiac chamber from which cardiac function can be assessed. In this system, we designed a custom algorithm to evaluate footage of heartbeats obtained from live embryos and to extract cardiac parameters for physiological analysis. Embryos used for zCAS analysis carry the *cmlc2:GFP* transgene that drives the expression of the green fluorescent protein (GFP) specifically in cardiomyocytes. As shown in Fig.1, the cardiac chambers of *Tg(cmlc2:GFP)* embryos are marked by GFP expression. Once a region of the heart is defined, the zCAS program generates a contraction profile to facilitate the quantitative measurement of heart rate and diastolic and systolic widths of the atrium and ventricle (Figure 1C,D). Cardiac parameters such as shortening fraction (SF), ejection fraction, stroke volume and cardiac output are then derived from these values to assess cardiac function. A steady increase in the heart rate of 2-, 3-, and 4-day embryos using zCAS is consistent with measurements previously reported, verifying the validity of zCAS (Figure 1E) [50,52].

As described above, we have found that treatments of amiodarone, fluspirilene, niguldipine, and pimozide disrupt normal cardiac contraction. We used zCAS to further evaluate the impacts of these compounds on the developing zebrafish heart. We found that embryos treated with pimozide and niguldipine displayed dilated atria and nearly silent ventricles two days after fertilization, and the hearts became silent by three days of development (n= 72/75 and 78/80, respectively; Fig. 2A–B). The SF of the atria of these embryos are not significantly different from untreated siblings, whereas the ventricular SF of embryos treated with pimozide or niguldipine are reduced to 5.6% and 10% of the levels of the untreated embryos, respectively, indicating that pimozide and niguldipine have more profound effects on ventricular contractility.

Treatment of fluspirilene and amiodarone on the other hand, caused cardiac arrhythmia. Most of amiodarone- and fluspirilene-treated embryos (n = 39/78 and 43/59, respectively) displayed a 2:1 AV block, a phenotype clearly noted by visual inspection. Interestingly, cardiac contraction profiles produced by zCAS uncovered other subtle defects. The atria of fluspirilene treated embryos displayed an alternating beat-by-beat rate (between 0.525 seconds/beat and 0.63 seconds/beat) resembling alternans in mammals (Fig. 2E–F). In addition, the atria of amiodarone- and fluspirilene-treated embryos exhibited an alternating contracting force of the left and right walls, where a contraction of the more forceful left atrial wall is followed by a contraction of a more forceful right wall (Fig. 2C–D, F). Furthermore, zCAS analysis revealed minor relaxation periods between each ventricular

beat in amiodarone-treated embryos using zCAS (Figure 2D). These observations demonstrate that zCAS is a sensitive and reliable high throughput tool, which can assist in evaluating the physiological impact of genetic mutations and small molecules on the embryonic heart.

Conclusion

Cardiovascular studies in zebrafish have primarily focused on the embryonic heart. Studies using zebrafish cardiac mutants and morphants have revealed insights into cellular and molecular mechanisms regulating embryonic cardiac contractility and rhythmicity. The ease of conducting chemical genetics screens in zebrafish, together with the advances in analytical technology such as zCAS, provide a cost-effective and reliable means for determining the effects of small molecules on the cardiovascular system. Success in combining chemical genetics with zebrafish mutants and/or morphants facilitates modifier screens for specific cardiovascular phenotypes and demonstrates the feasibility of using this approach to elucidate molecular pathways critical for regulating cardiovascular functions as well as to discover new therapeutic agents. One remaining question is whether the fish model could be utilized for investigating adult cardiac disorders. Recent studies showed that adult fish heterozygous for the KCNH2 mutation have prolonged QT interval similar to the dominant trait observed in humans [21], suggesting that the study of adult heterozygous fish may afford new insight into the progression of cardiac disease. Developing technologies required for imaging and physiological studies in adult fish will greatly facilitate these lines of study.

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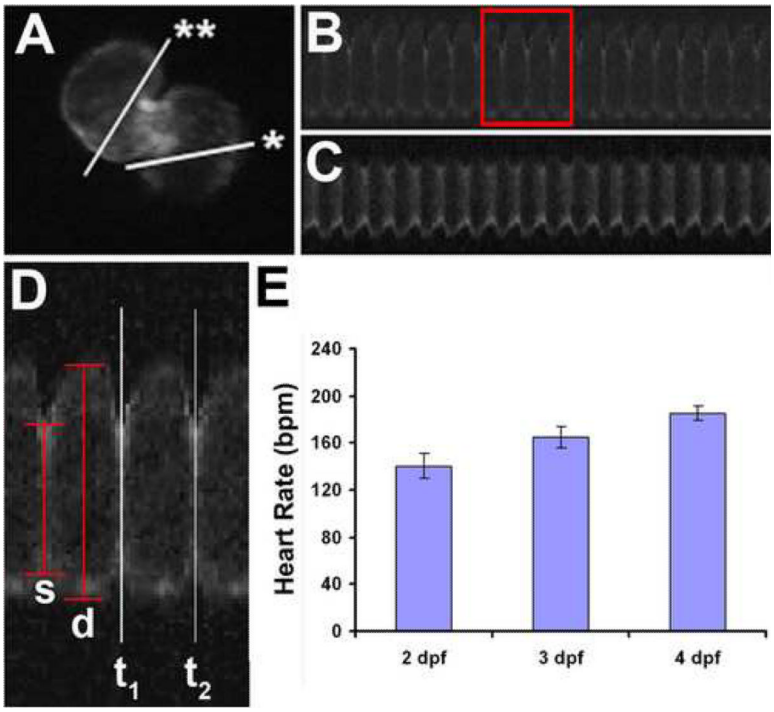

Figure 1.

zCAS analysis for embryonic fish cardiac function. (A) *Tg(cmlc2:GFP)* heart of 2 dpf zebrafish embryo. Asterisk and double asterisk mark regions of the atrium and ventricle scanned. (B) Representative atrial cardiac contraction profile. (C) Representative ventricular cardiac contraction profile. (D) Magnified view of atrial contraction from (B). Heart rate determined by measuring time between two systolic phases, t_1 and t_2. D: end-diastolic diameter, s: end-systolic diameter. (E) Heart rate measured by the zCAS program during fish development. dpf: days post fertilization.

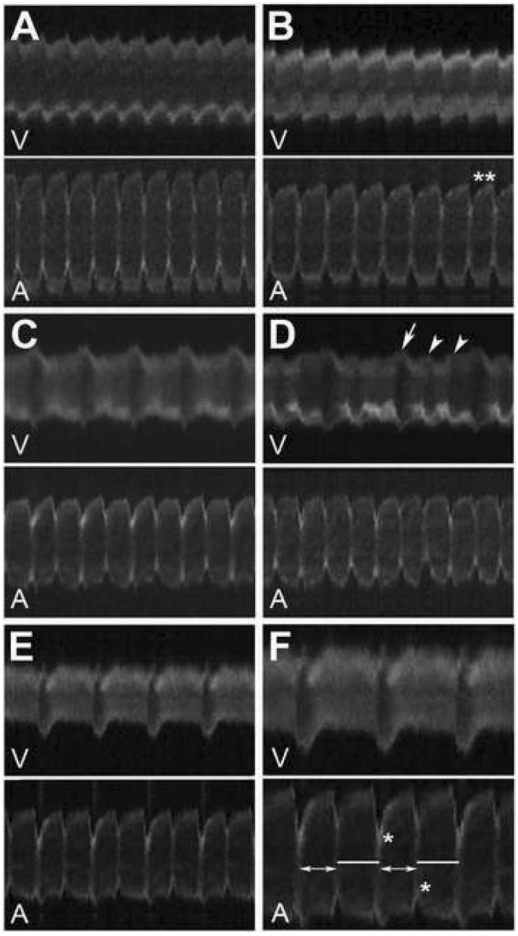

Figure 2.

Cardiac contraction profiles of chemically-treated *Tg(cmlc2:GFP)* zebrafish embryos at 2 dpf. (A) Niguldipine-treated embryos; (B) Pimozide-treated embryos, double asterisk (**) mark anomaly in interval between contractions; (C, D) Amiodarone-treated, white arrow and arrowheads mark phases of major and minor relaxation, respectively; (E, F) Fluspirilene-treated, lines and double headed arrows denote relaxation periods alternating between 0.63 and 0.525 s, respectively. Maximum atrial wall contraction is marked by an asterisk (*). V: ventricle line scan; A: atrial line scan.

Table 1

Selected zebrafish mutants with cardiac contractility and/or rhythmic defects

Zebrafish mutant	Gene	Cardiac Phenotype	Ref
Contractility			
tell tale heart	*cardiac myosin light chain 2*	Weak or no contractility; loss of myofibril assembly; extended AV canal region	8
pickwick	*titin*	Poor contractility; disrupted sarcomeres	11
silent heart	*cardiac troponin T*	Non-contractile heart; disorganized sarcomeres	12
weak atrium	*atrial myosin heavy chain*	Weak atrial contractility; disorganized myofibrils; thickening of ventricular wall	13
T2EGEZ8	*tropomyosin4*	Non-contractile heart; disorganized sarcomeres	50
cardiofunk	*actin, alpha, cardiac muscle 1*	Chamber dilation; blood regurgitation	51
main squeeze/lost contact	*integrin-like kinase*	Deterioration contractility; blood vessel dilation	24,25
dead beat	*phospholipase C γ1*	Deterioration ventricular contractility; lacks lumenized vasculature	52
Rhythmicity			
island beat	*L-type calcium channel subunit α1C*	Uncoordinated contraction	15
tremblor	*Na/Ca exchanger 1*	Disorganized myofibrils; uncoordinated contraction	16,17
heart-and-mind	*Na/K ATPase α1B1*	Dysmorphic heart tube; uncoordinated contraction	18
breakdance	*kcnh2*	2:1 rhythm (loss-of-function)	19,20,42
reggae	*kcnh2*	Intermittent cardiac arrest (overactivation)	21
futka	*connexin 36.7/early cardiac connexin*	Defective sarcomeres; arrhythmia	53
santa	*cerebral cavernous malformation 1*	Loss of concentric growth of ventricle; absence of AV conduction delay	3,54
valentine	*cerebral cavernous malformation 2*	Loss of concentric growth of ventricle; absence of AV conduction delay	3,54
slipjig	*foxn4*	Failure to loop; lacks AV conduction delay	26
hobgoblin	*tcf2*	AV block	3