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Danio rerio as a Momentous Model of Cardiovascular Research: A Review

Ivy Ghosh[1]*, Samrat Bose[1]

**[1]Guru Nanak Institute of Pharmaceutical Science and Technology.
157/F, Nilgunj Road, Panihati Sodepur,Kolkata-700114, India**

Abstract : Cardiac development is a composite process which may include cell specification and differentiation along with detailed morphological study. Combinatorial application of optical clarity along with large scale of mutant makes the zebrafish most important vertebrate model for the identification and justification of various cardiac problems. *Danio rerio* has become a robust exemplanary to elucidate the basic molecular and cellular mechanisms and genetic approaches of cardiac development and function. Origin of the cells of the cardiac progenitor can be analyzed in this oviparous model and the formation of the heater tube can also be examined by the process of serial sectioning of the embryos. Summarization of the knowledge of the recent discovery of second heart field of zebrafish has been discussed in this review. Comparing with the humans, zebrafish has given a wide range of information to express and to study the human disease related gene variants which helps to identify the mechanisms of the disease. In this review we mainly focus on the cardiac developmental study and specification in which zebrafish has been applied as a model system in human congenital and acquired cardiac diseases.

Keywords : Zebrafish, Cardiac outflow, Trabeculation, Valvulogenesis, Conduction system, Congenital, Cardiomyopathy.

A Review

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➢ Introduction:

Cardiac disease has become a foremost cause of morbidity and mortality and many of these problems emerge from the congenital defects which effect the development and maturation of the heart. [1, 4] Heart is a flawless system for both the approaches of embryological and genetic basis.[2] The *Danio rerio* has become an apparent robust exemplanary to unravel the cardiac development based on genetic, molecular and cellular mechanisms. According to the study of Stainier and Fishman in 1993, the development of heart at the embryonic stage of this vertebrate model is very much prominent and can be easily scored.[1] The cardiac system of this tropical fresh water fish represents as prototypic heart containing a single atrium and ventricle.[6] During the early embryogenic stages of this zebrafish model, the origin of cardiac progenitors can be analyzed and the observation of the separating lineages of the atrium and ventricle can be done by injection of single blastomers by using lineage tracer dye.[2, 3] According to the study of Weinstein and Fishman in 1996 and Fishman et al. in 1997, the molecular mechanism of cardiac patterning of this oviparous model is indistinguishable to the more complex patterning of higher vertebrates cardiovascular system.[6]Zebrafish affords various well defined advantages as an exemplanary in the research work of cardiac developmentary evolution. Firstly, due to the external progress of the embryos permits an undeviating noninvasive surveillance of the heart development. Secondly, cardiac mutants of this vertebrate model remain alive and may continue to develop for several days, as a result a circumstantial analysis of their phenotype can be possible.[5] Thus, the studies of cardiac development using *Danio rerio* as a vertebrate model organism will become a challenging work for the purpose of large-scale screening of the active cardiovascular substances , particularly for the new drugs.[6]

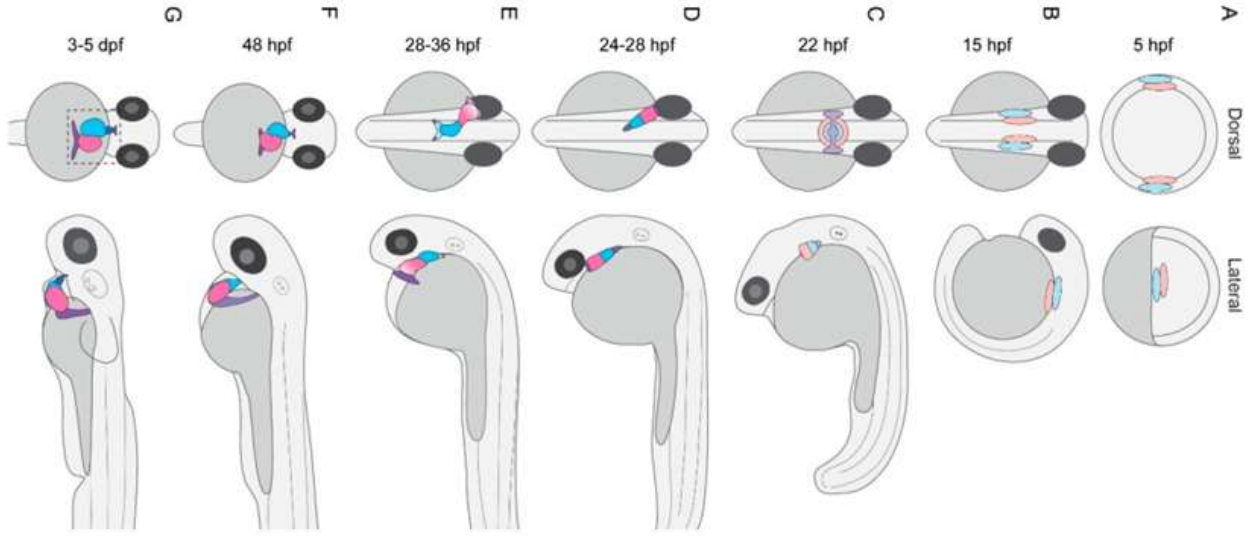

Fig 1: Stages of cardiac development in zebrafish embryos in ventral view.[11]

➢ Cardiac Development:

As zebrafish is a rapidly developed tinny vertebrate model, after 24 hours post fertilization (hpf) the fertilized eggs of this oviparous exemplanary has grown up into an embryo. Then the embryo may inaugurate various organ commencements which may be inclusive of nervous system and heart tube with contraction followed by blood circulation. [1] Anatomical structure of this fresh water model consists of four chambers i.e. Sinus venosus, atrial cardiomyocytes (CMs), ventricular cardiomyocytes (CMs)[11]and bulbous arteriosus. [1, 8]

- **Cardiac Outflow-**

The outflow tract (OFT) of the *Danio rerio* consists of bulbous arteriosus and aorta. The bulbous arteriosus is comparable to the conotruncus of mammals and is made of the inner endothelial cell layers which are lined by thick smooth muscle cells. The desaturated venous blood can be pumped to the ventral aorta which leads to the gill arches where oxygenation occurs and from where it can be circulated to the whole body.[10]

- **Heart Muscle Growth-**

During the development of heart, the atrium persists a thin layer of myocardial cells whereas the ventricle remains a thick layer of trabeculae which are essential to originate adequate contractile force to significant circulation in the developing embryo.[1] At this stage myofibrilogenesis is accomplished by thin and thick filaments, M line and Z discs which are associated into equal arrays of cardiac sarcomeres. [30]The size of adult zebrafish is 1mm or larger in ventricular length. The atrium is well ordered internally with pectinate muscle and the ventricle is composed of a closely arranged layer enveloping the inner layer of trabeculae. [1,12]

- **Trabeculation-**

Cardiac trabeculae are highly systematic and luminal. Muscular ridges are fringed by endocardial cells in the ventricular lumen. Trabeculae expand surface area of myocardium for the purpose of oxygenation of blood and are censorious for cardiac function. Thetrabecularmyocardium growingly increases in the developing heart. Due to the maturation of cardiac wall, the trabeculae endurea large-scale improvement in alliance with compact myocardial proliferation and as well as the formation of the coronary vasculature and developmentation of the conduction system. [10]

- **Valvulogenesis-**

In the development of the vertebrate heart, cardiac valves are the significant component. The main functions of the valves are to confirm the unidirectional blood flow in the system and restrict the retrograde flow. [13, 14]The malformation of valves is the cause of many human congenital problems and adult-onset cardiac problems. The atrioventricular (AV) canal is originated in between the border line of the atrium and ventricle which can be easily identified during looping morphogenesis. At 40 hpf, AV CMs increase their luminal surface as well as also decreases their abluminal surface.[16] Around 76 hpf, the cells of Av endocardium experiences a transition of epithelial-to-mesenchymal (EMT) [15]which permits the overall prevention of retrograde blood flow. [1, 10]Zebrafish bmp4 is expressed in the AV canal, and Bmp signaling activity regulates expression of the tbx2b in the AV canal myocardium. [7]

(a) At 15 hpf, cardiac precursors move towards the anterior lateral plate mesoderm. (b) Starting of cardiac fusion at 18 hpf. (c) Formation of cardiac cone after cardiac fusion at 21 hpf. (d) In between 26 hpf and 48 hpf cardiac looping of heart tubes occurs. Bending of the linear heart tube occurs and formed a S-shaped loop. (e) The rotation of heart tube occurs.

According to Clark in 1990, during the period of embryogenesis, heart is the primarily major organ to develop and become functional. According to Manasek in1970, before the completion of the connection of circulation the first heart beat of the embry ocan be observed. The development of the heart of the *Danio rerio* revenues through various closely kindred steps as amniotes. The precardiac cells specification in the anterior lateral plate mesoderm (ALPM) [1, 6] is the beginning stage of heart development which can be subsequent to bilateral heart fields at the midline of the embryo. Mechanism of various cardiac events at the primordial stage of embryo can be clearly understood due to powerful genetic accountability and the capability of high resolution bio-imaging of the embryos.[1]

Table 1: Comparison of Early Heart Development with Mouse andZebrafish[1]

Cardiac Events	Mouse	Zebrafish
❖ Migration of precardiac cells from epiblast	7 dpc (primitive streak)	50% epiboly (5.5 hpf)
❖ Primordial assembly of myocardial plate	7 dpc (late primitive streak)	8-10 somites (~13 hpf)
❖ Initial generation of single heart tube	8 dpc (5-10 somites)	20 somites (~19 hpf)
❖ Begins contraction of tubular heart	8.5 dpc (8-10 somites)	26 somites (22 hpf)
❖ Looping	8.5 dpc	33hpf
❖ Formation of endocardial cushions	9.5 dpc	48 hpf

Due to the involvement of the cell specification as well as tissue remodeling zebrafish provides distinctive advantages of cardiac development and functions for exploring genetic and molecular mechanism because the embryos of the *Danio rerio* are flexible to Whole In-situ hybridization (WISH). [6, 9, 19]Comparing with the mouse embryos, the direct observation of the heart development and function can be visualized easily under stereomicroscopy in early embryonic period. [6] At 5 hpf which is the stage of just before gastrulation period the formation of primary germ layers such as ectoderm, mesoderm and endoderm starts due to the specification of cardiac progenitors. By 5 hpf, fate mapping studies can identify the heart chambers. The size of the heart chambers, their morphology and contractile properties can be confirmed by using various sarcomeric proteins i.e. atrial myosin heavy chain (*amhc*), in the atrium versus ventricular myosin heavy chain (*vmhc*) in the ventricle.[1, 8]At the 16 hpf a cardiac cone can be created due to the fusion of bilateral heart fields which denotes the markers of cardiac distinction such as sarcomeric genes tropomyosin and troponin.Then the anterior expansion of cardiac cone occurs and as a result the primary formation of heart tube may be moderately done. By 24 hpf the primordial heart tube consists of an outer myocardial and an inner endocardial layer which is separated by an extracellular matrix named as "cardiac jelly".[18] After the formation of this extracellular matrix, the linear shaped heart tube gains the capability to contract in peristaltic rhythm by maintaining a conduction velocity of 1mm/sec. [1,17] According with the mechanical structure the electrical activity is one of the most important property of the development of heart and zebrafish can posses its own advantages in this regard. Daniela et al. recognized an electrical coupling gradient by using high-speed optical mapping of transmembrane potentials and calcium concentration across the progressing ventricular myocardiumin isolated embryonic hearts of *Daniorerio*.[17] According to David et al. a zebrafish exemplanary of cardiac repolarization can be proposed by using fluorescent reporters of transmembrane potential which also can establish a conduction of drug-sensitized genetic screening function in this vertebrate model.[6, 21] The outer curvature (OC) of the myocardial and endocardial cells are elongated, bigger in shape and may conduct electrical activity three time faster than the cells of the inner curvature (IC). [6, 8]At 33 hpf, heart tube in loop is formed which may be 'S-shaped' can displace ventricle to the right of the atrium. By 36 hpf, transition may occur from slow peristaltic wave to sequential contraction of the chambers which denotes the function of the onset of Cardiac conduction system (CCS). [1] At 48 hpf, the main components of heart have formed but the heart remains still immature and deficient of auxiliary cells and some other supplementary structures that are essential for the function of various organisms. [10]

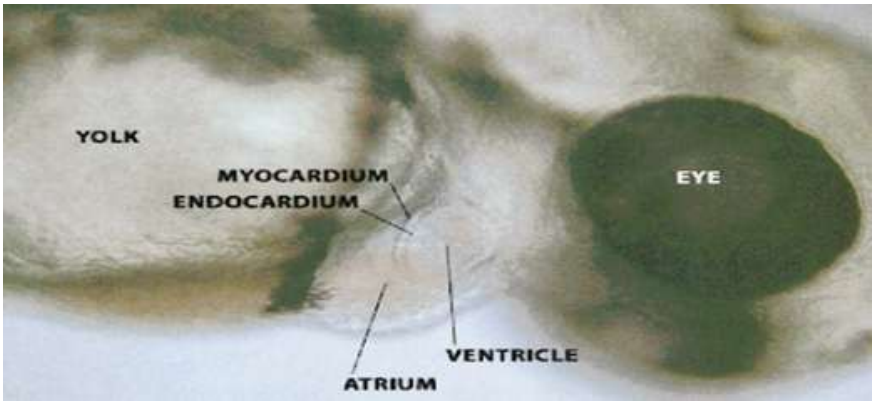

Fig 2: Lateral view of heart chambers and layers of 2-day old Zebrafish embryo[8]

- **Early Cardiac Specification and Differentiation in Zebrafish:**

Organogenesis is a facile and composite developmental process. Embryogenic organ formation includes the specification and differentiation of various cell lineages. [8] Heart is the primary formed and functionally active organ in the vertebrate embryo. [7,8] Development of heart begins with the specification of the progenitor cells of the myocardial and endocardial cells.[3] In all cases of vertebrate models, the bilateral arising occurs in the myocardial progenitors within the anterior lateral plate mesoderm (ALPM). [8] The progenitor cells of myocardium consist of two pools i.e. atrial and ventricular. According to Stainier et al in 1993, the ventricular progenitor cells are located laterally in the marginal zone at the embryonic side before initiation of gastrulation or the early blastula period (512-cell stage). [3,7, 8]Comparing with the above the atrial progenitor cells situated more ventrally in the marginal zone than the ventricular progenitor cells. According to Warga and Nüsslein-vol-

hard in 1999, in the late blastula period (40% epiboly) the myocardial progenitors are noticed within the three tiers of blastomers which is nearest to the marginal region of embryo. [8]The size of the pool of the myocardial progenitor can be prevented by the signaling of retinoic acid. [7] Keegan et al. noticed that by using a combinatorial effect of genetic, pharmacological and fate mapping techniques the adoption of cardiogenic fate due to the potential of the native cells can be restricted by retinoic acid (RA) signaling.*aldh1a2*(*aka neckless*) are the defective mutants in RA synthesis which can produce a huge number of cardiomyocytes. For the explanation of the RA signaling in cardiac specification more prominently, generation of the high-resolution fate map in the lateral marginal zone of the late blastula period is very much necessary. [9]*Isl1* the mutant of zebrafish embryo posses a remarkable moderate decreasing effect in the cardiogenic differentiation only at the venous pole. Zebrafish*Isl1* has various functions at the cardiac region. [7] According to Serbedzija et al in 1998 and Yelon et al. in 1999, in most cases of this vertebrate model *nkx2.5*-expressing cells terminally distinct into myocardiocytes. [8, 9]As the study of Reiter et al. in 1999 and Yelon et al. in 1999, differentiating myocardiocytes begin a gene expression which may encode sarcomeric proteins of cardiogenesis such as *cmlc1* and *cmlc2*.[8]

- **Heart Field in Zebrafish:**

Researchers have been proposed that the development of the heart of the vertebrate model occurs through the participation of two sets of distinct precursor cells termed as First Heart Field (FHF) and Second Heart Field (SHF). First heart field cells help in formation of the left ventricle whereas the Second heart field contributes in shaping of right ventricle, inflow outflow of tracts andsome portions of aorta. The FHF mainly contributes to the formation of the linear heart tube. [10]

- **Secondary Heart field-**

Supplementary cardiac cells are appointed to the cardiac tube in a secondary wave of differentiation which is called as SHF which extends the linear heart. [10]The cells of the SHF help in the further progress of the growth of the heart tube.[3] Cells of the SHF also help in the elaboration of the functional pumping organs. [5]Scientific studies have noticed that SHF progenitor cells lie medially in the splanchnic mesoderm. Cardiomyocytes (CMs) are derived from the progenitor cells of SHFwhich contributes to the arterial and venous poles of the heart tube. [5, 10] The complete expression of SHF can be expressed by homeobox gene *Islet-1* which is the mutant or regulator of the development of SHF. The cells of SHF are situated dorsally to the primitive heart tube in the pharyngeal mesoderm. Second Heart Field cells also produce an improvement of craniofacial muscles. The presence of two-septated chambers of heart and a primary outflow tract (OFT) ensures the presence of SHF in the *Danio rerio* model.[1, 5,20,23]

- **Cardiac Conduction System:**

A functional cardiac conduction system (CCS) is essential to commence, maintain and correlate cardiac rhythm as result synchronized contractions occur. Large-scale screening of gene mutation influences the CCS which helps in detection of four developmental stages in zebrafish. There are 17 identified mutants which are responsible for the detection of the defects of the CCS.[22]

- **Stage1-** It begins in between 20-24 hpf, when a wave, which is linearly activated, transmits across the heart tube[3] from the sinus venosus to the outflow tract (OFT).
- **Stage 2-** At 36-48 hpf, a remarkable AV conduction delay improves.
- **Stage 3-** In between 72-96 hpf, an undeveloped rapid conduction network progresses within the ventricle.
- **Stage 4-** This rapid conduction network develops entirely and as a result an apex-to-base activated pattern is appeared.

The pacemaker activity is significantly present in this vertebrate model and produces equivalent activity to the mammals. A mutant, *slow motion*, indicates the function of defective pacemaker current. A recent research work ensures that knockdown of *shox 2* produces pronounced sinus bradycardia. The *isl1* genes in zebrafish and as well as mammalian heart can be expressed in the cells of sinoatrial pacemaker. In *Danio rerio*, *isl1* gene expression distinguishes the whole pacemaker population which forms a ring-like structure at the margin between the sinus venosus and atrium. [1, 7, 24]

- **Future Aspects:**

Characterization of the mutants of the zebrafish model and the cloning of the genes which are affected has given the focus on various aspects of the regulation of the patterning of the heart. According to the study of Sunnerton and Weller in 1997, morpholinos are extremely stable antisense oligonucleotides which can be designed to lessen translation of genes which are desired. So according to Nasevicius and Ekker in 2000, the injection of morpholinos gives an expeditious method to examine the developmental function of a gene in this vertebrate model. This strategy will be very accommodating in investigating potential regulatory genes which are demonstrated in the heart of zebrafish. Future studies of using the emerging technologies can extend the proper understanding of the myocardial patterning. [8]

Danio rerio model has been rapidly developed and become an extraordinary significant model organism for cardiovascular research. There are only few areas of cardiovascular research work where the potentiality of this vertebrate model has not yet been proved. The principle challenge of this oviparous model is its similarity or closeness of the human cardiovascular pathology.Recent developments in the technical field permit the generation of mutants for any gene.As a result, in near future there has an availability of gene mutants. Therefore, the cost of determination of phenotype severity of a particular mutation becomes lower than mice model. Current development in mass screening approaches such as chemical genetics will be a fundamental area of zebrafish model.[1]

- **Zebrafish Model for Congenital Heart Defects-**

Current approaches is involved in discovering the human congenital heart defects (CHD) include pedigree studies and candidate screens. More currently, reverse genetic approaches have been used to distinguish single nucleotide polymorphisms (SNs) in candidate genes.[25,26,27] In this approach, theselections of the candidate genes are typically are done from pre-existing knowledge which can be obtained from fundamental scientific studies using various model organisms. This knowledge can be originated from this vertebrate model, in which mutations where distinguished in the Nodal co-receptor EGF-CFC/CRYTC in patients who has been suffering from severe congenital visceral laterality defects. [28] Expression of the patient-specific ALK2 L343 variant in zebrafish explained that this results in a malformation of the AV canal and compromised expression of endocardial cushion markers.[7,10]

- **Cardiomyopathies-**

Human Cardiomyopathies are diseases that mainly affect the myocardium. The two forms are dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM). [29] Various mutants of zebrafish have been identified which exhibit heart phenotypes resembling the features of human Cardiomyopathies and their individualization have provided a better apprehension of the human disease.[7]

Conclusion-

The zebrafish heart may furnish a flawless system for the combining study of embryological and genetic perspective of vertebrate organogenesis. In this review, we have established the outline knowledge of cardiac development. *Danio rerio* is a modernistic disease model in cardiac problem. Now a days, this oviparous model has been used to study the main mechanism of action of human cardiac disease such as acquired cardiac disease and also human congenital cardiac problems. This vertebrate exemplanary provides some definite advantages for the heart developmental study because of its availability in embryology and genetic approaches. Due to the large scale genetic screening and identification of various novel factors as well as regulatory mechanisms such as migration of cardiac progenitor cells, cardiogenic specification and differentiation, morphogenesis ofvarious heart organs this model provides large development in human cardiac study. It can be predicted that in upcoming days, this field of research will progress in rapid manner due to the growing interest of the researchers in cardiac diseases and also due to the ease of availability of this oviparous vertebrate model.

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Authors:

Ivy Ghosh[1], Samrat Bose[1]

[1]Guru Nanak Institute of Pharmaceutical Science and Technology.
157/F, Nilgunj Road, Panihati Sodepur,Kolkata-700114

Main Author: Ivy Ghosh.

Email id: ivyghosh1994@gmail.com

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