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Zebrafish as a tractable model of human cardiovascular disease

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ABSTRACT.

Mammalian models including non-human primates, pigs and rodents have been used extensively to study the mechanisms of cardiovascular disease. However, there is an increasing desire for alternative model systems that provide excellent scientific value while replacing or reducing the use of mammals. Here we review the use of zebrafish, *Danio rerio*, to study cardiovascular development and disease. The anatomy and physiology of zebrafish and mammalian cardiovascular systems are compared, and we describe the use of zebrafish models in studying the mechanisms of cardiac (e.g. congenital heart defects, cardiomyopathy, conduction disorders, regeneration) and vascular (endothelial dysfunction and atherosclerosis, lipid metabolism, vascular ageing, neurovascular physiology and stroke) pathologies. We also review the use of zebrafish for studying pharmacological responses to cardiovascular drugs, and describe several features of zebrafish that make them a compelling model for *in vivo* screening of compounds for the treatment cardiovascular disease.

INTRODUCTION

The purpose of this review is to provide an overview on the use of zebrafish models of cardiovascular physiology and disease. We compare the anatomy and physiology of zebrafish and mammalian systems and briefly overview the advantages of zebrafish models in terms of gene modification and unique imaging approaches. The manuscript then summarizes zebrafish models of cardiac pathology with emphasis on congenital heart defects, cardiomyopathy, conduction disorders and regeneration. We then summarize zebrafish models of vascular pathology with focus on endothelial dysfunction and atherosclerosis, lipid metabolism, vascular ageing, neurovascular physiology and stroke. It should be noted that we have not have not referenced every zebrafish study within these subject areas for the sake of brevity. We also highlight many examples where cardiovascular research has combined pharmacological and genetic approaches, summarize recent technological advances and how they can drive future research in cardiovascular disease.

ZEBRAFISH AND MAMMALS: COMPARATIVE PHYSIOLOGY

The heart

Due to having a single circulatory system, zebrafish have a 2-chambered heart comprised of a single atrium and a single ventricle, connected to the circulatory system by the bulbus arteriosus which regulates blood pressure to the gill vasculature. In addition to the lack of chamber septation and the presence of the teleost-specific bulbus arteriosus, adult zebrafish hearts are also hyper-trabeculated compared to endothermic vertebrate hearts, with comparatively little compact myocardium in the ventricular wall[1]. Despite the relative simplicity of the zebrafish heart when compared to mammals, most of the specialised cell types and structures (for example pacemaker, atrioventricular valve, aortic valve, trabeculae, coronary vasculature), and contributing cell types (myocardium, endocardium, epicardium, cardiac neural crest, second heart field, fibroblasts) are conserved. Similarly, the signalling pathways and morphogenetic processes driving early heart development are well conserved[2], and zebrafish are now widely used as a model to investigate cardiac development and morphogenesis.

Zebrafish are increasingly employed to analyse disorders of cardiac function. Heart rate in zebrafish is more similar to human than those of other model organisms, and ECG analysis of adult zebrafish identifies clear P, QRS and T waves, with a QT duration that indicates a comparable repolarisation time[3] demonstrating that electrophysiology of the adult zebrafish heart is highly similar to human[4-6]. Despite the small size of embryonic zebrafish hearts, techniques for measuring ECG profile in embryos have been developed[7], identifying similar ECG features from 3dpf onwards as those observed in adult zebrafish. Since zebrafish are ectotherms, these electrophysiological dynamics are temperature dependent, and thus it is important that comparative analyses of electrical activity in the zebrafish heart are temperature controlled. The similarities in ECG activity between zebrafish and human hearts, in particular the distinct QT interval, are due to a highly comparable ventricular action potential (AP), with both zebrafish and human exhibiting a long plateau phase at positive voltage during repolarisation. Many of the key ion channels that govern AP dynamics in

human have orthologs in zebrafish, for example the Na+ channel-encoding *SCN5A,* the inward rectifier $I_{K,ACh}$ K+ channel-encoding *KCNJ3* and *KCNJ5*, and the outward rectifying I_{Kr} K+ channel-encoding *KCNH2*. However, there are also key differences between human and zebrafish AP dynamics and channel composition or function, with atrial AP less similar between species, different composition of the inward rectifier I_{K1} channel, and absence of delayed rectifying current I_{Ks}-encoding genes (reviewed in[8]) While these similarities make the zebrafish a highly suitable model to understand human cardiac electrophysiology, the variation in ion channel composition and slight differences in AP dynamics should be kept in mind during pharmacological analyses.

Vascular system

The basic structure of vascular anatomy is highly conserved between zebrafish and other mammalian models, including humans [9]. The unique advantage of the zebrafish model is that due to the optical transparency of the embryos and larvae, the morphological and functional changes of blood vessels can be observed noninvasively in living animals. Developmental processes involved in vascular development, including vasculogenesis, angiogenesis and vascular remodelling are comparable between zebrafish and mammals, requiring tight regulation of the same key molecular pathways [10]. The main axial vessels, the dorsal aorta and posterior cardinal vein form by vasculogenesis starting from around 10 somite stage (14 hours post fertilisation (hpf)) from the progenitors in the lateral plate mesoderm. The intersomitic vessels of the trunk which are among the first angiogenic vessels to form in vertebrates, start sprouting from the dorsal aorta from around 22 hpf. Circulating blood cells can be observed in the main axial vessels shortly after the onset of heart contractions at 24 hpf. Even though the cardiovascular system is one of the first to form during development, the zebrafish embryo obtains oxygen by passive diffusion from water for the first several days. This feature enables studies of experimental manipulations and cardiovascular mutant phenotypes which would be lethal in humans and other mammals.[11]

Important differences to be noted between fish and mammals include the relative sparsity of mural cells associated with the endothelium as well as low arterial blood pressure (0.3-0.4 mmHg in larvae and 1.5-2.15 mmHg in adult zebrafish).[12] Nevertheless, there seems to be a conserved response of the vasculature to the classical vasodilators and vasoconstrictors, as well as to a number of cardiovascular drugs [13, 14]. Treatment of zebrafish larvae with nitric oxide (NO) donor sodium nitroprusside resulted in a significant increase in both arterial and venous vessel diameters, whereas application of NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) led to a significant decrease in vessel diameters, which corresponds to responses in mammals. Similarly, in larvae pre-treated with L-NAME to inhibit endogenously produced NO, addition of epinephrine resulted in vasoconstriction [13]. A comparative analysis of the *in vivo* cardiovascular responses of zebrafish, rat, dog and human to 3 cardiovascular drugs which modulate beta-adrenergic and renin-angiotensin systems (propranolol, losartan and captopril) showed that zebrafish and human responses were

largely comparable in >80% of drug/endpoint combinations, demonstrating the translational power of zebrafish [14].

ZEBRAFISH MODELS: UNIQUE GENETIC AND IMAGING SYSTEMS

The use of zebrafish as a model to study cardiovascular function or disease has accelerated in recent years, with a literature searches retrieving in excess of 5000 publications since 2010. This rise in popularity can be attributed to several advantages the zebrafish has over mammalian systems, primarily, the potential for examination of the formation and function of the cardiovascular system non-invasively in a live organism. Its small size, optical translucency, high fertility, rapid development and affordability make the zebrafish an attractive model to study human disease. Moreover, analysis of early zebrafish embryos can in many instances replace studies of rodents and other mammals thereby reducing the use of more sentient animals in research. Approximately 70% of human genes share an orthologue with zebrafish and 82% of known human disease genes are also present in the zebrafish genome.[15] This high degree of conservation coupled with its amenity for genetic manipulation has positioned the zebrafish at the forefront of biomedical research.

The zebrafish genetic toolbox. One of the major advantages of using zebrafish in biomedical research is the ability to perform genetic modification with relative ease. This technology has facilitated temporal and spatial control of gene expression allowing both gain-of- and loss-of-function genetic studies. It has been applied extensively to create transgenic lines with fluorescently labelled cells allowing cell behaviour to be studied in unparalleled detail. A wide range of methods are available for disrupting gene function in zebrafish for reverse genetic studies. However, these methods have now largely been replaced by CRISPR-Cas9 mediated gene editing which is cheaper and simpler to implement. In addition, gene expression can be transiently inhibited throughout the embryo at the message or protein level using morpholino oligonucleotides (MOs) or in specific tissues using CRISPR-Interference (CRISPRi)[16]. Genetically, zebrafish have undergone a partial genome duplication, which can make studying the role of certain genes more challenging due to functional redundancy of paralogous genes. Alternatively, this redundancy can also be advantageous in some contexts because later functions of some genes which are embryonic lethal in mammals can be uncovered in zebrafish.

The imaging toolbox.

The zebrafish cardiovascular system can be visualised using a multitude of approaches which range from traditional *in situ* hybridization in fixed tissues to real-time analysis of transgenic reporter lines, which express fluorescent proteins in cardiac and vascular-specific cell types. In recent years, fluorescence microscopy techniques have shifted towards higher resolution, increased tissue penetration depth, and a reduction of image acquisition artefacts. While confocal and AiryScan microscopy are often used to achieve increased *in vivo* resolution, light sheet fluorescence microscopy (LSFM) has become a crucial image acquisition method,

allowing data acquisition at a greater anatomical depth and imaging duration from hours-to-days [17]. Of particular relevance, visualization of cardiac dynamics has received increasing attention, allowing for 3D (+time) data acquisition and analysis, using gating approaches in imaging and post-processing [18] . Additionally, the ability to control cardiac function optogenetically[19] and analyse endothelial cell calcium dynamics [16] has opened up new avenues for cardiovascular studies.

CARDIAC DISEASE MODELS

Zebrafish have been used to model multiple aspects of cardiac development, function, and disease. Here we highlight specific examples where zebrafish have made substantial contributions to our understanding of cardiac defects, along with the genetic and experimental techniques used to generate and interrogate these models.

Congenital heart defects (CHD) are the most common birth defect, affecting approximately 1% of live births worldwide, and comprise a spectrum of structural malformations, including septal defects, inflow and outflow tract malformation, chamber hypoplasia, and valve dysgenesis. Despite the comparative simplicity of the zebrafish two-chambered heart when compared to the four-chambered heart of mammals with dual circulatory systems, the molecular regulation and morphogenetic processes underlying zebrafish heart development are highly conserved. Here we highlight specific examples where zebrafish have provided novel insights into CHD aetiology.

Heterotaxia syndrome is caused by perturbations in left-right patterning of the body during embryogenesis, resulting in loss of concordance of organ lateralisation, and disruption to internal asymmetries within organ systems. The heart is a highly asymmetric organ, and correct asymmetric morphogenesis is vital for correct cardiac function: individuals with heterotaxia often present with CHD. Key players in the pathways regulating early establishment of embryonic asymmetry are well established, with zebrafish mutants exhibiting heterotaxia phenotypes similar to those present in patients with mutations in the homologous genes (Figure 1). Recent studies identified novel variants in candidate genes potentially causative for heterotaxia [20 21], with functional validation through CRISPR-Cas9-mediated embryonic mutagenesis confirming *dnah10*, *rnf115*, *flna*, *kif7,* and *kmt2d* to regulate cardiac asymmetry [20 21]. Zebrafish mutant models have also shed light on the mechanisms underlying CHDs in individuals carrying mutations in *CFAP53*, encoding a ciliary protein [20, 21 22]. The genetics underlying CHD phenotypes are complex, and this highlights the value of zebrafish in dissecting the pathways and interactions that underlie cardiac morphogenesis.

Zebrafish represent an excellent model to investigate the impact of environmental factors on cardiac development. PM2.5 is fine particulate matter, high levels of which are found in urban areas with poor air quality/high levels of pollution. PM2.5 has been associated with increased

incidence of CHD and exposing zebrafish embryos to PM2.5 resulted in developmental heart defects, including heart malformations and bradycardia [23-27]. This model has been exploited to identify compounds which ameliorate the impact of PM2.5 on the heart, such as Folic Acid. Protective effects of Folic Acid from developmental heart defects has also been described in a zebrafish model of Fetal Alcohol Spectrum Disorder [28], which demonstrated the negative impact of continuous alcohol exposure during early development on cardiac morphology and growth. Zebrafish have also been used to model the impact of diabetes/hyperglycaemia during pregnancy on cardiac development [29]. Together, zebrafish can model the mechanisms underlying CHDs with either genetic or environmental origins.

Cardiomyopathy represents a spectrum of heart diseases encompassing structural changes of the myocardium, comprised of three main classes. Dilated cardiomyopathy (DCM) is a progressive disease characterised by an enlarged, often left, ventricle, thinning of the myocardial wall, and reduced cardiac output. Hypertrophic cardiomyopathy (HCM) is associated with a thickening of the ventricular wall. Arrhythmogenic cardiomyopathy (ACM) involves degeneration of cardiomyocytes, and gradual replacement with fibrofatty scar tissue, impacting heart function and leading to structural heart remodelling. Typically, cardiac function is progressively impaired, often leading to heart failure. Hallmarks of cardiomyopathy and heart failure are conserved in zebrafish, including the upregulation of genes associated with cardiomyopathy and heart failure, such as *nppa*/*nppb* [30]. Thus, studies in zebrafish have contributed significantly to our understanding of the mechanisms underlying cardiomyopathies through functional validation of novel cardiomyopathy candidate genes (Figure 2). For example, a 2015 study describing transcriptomic analysis of embryonic and adult zebrafish hearts identified zebrafish homologues for 49 out of 51 DCM-associated genes [31].

TITIN (TTN) encodes a giant sarcomeric protein containing binding sites for a number of proteins along its length, which associate with the Z-disc, I- and A-bands, and M-line regions of the sarcomere. Mutations in *TTN* result in both DCM and skeletal myopathy, and may account for as many as 25% of DCM cases [32]. However the location of mutations within the *TTN* gene appear to influence disease severity, with mutations in the C-terminal more likely to be associated with profound DCM [32, 33]. Targeted CRISPR-Cas9 mediated mutagenesis in zebrafish has shed light on why the position of truncating mutations within *TTN* may impact disease severity [34]. In the latter study, the authors generate 6 different truncating mutations in the zebrafish *ttnb* gene (which most resembles human *TTN*), resulting in truncations either in the z-disc, proximal/mid I-band, or distal A-band domains. While all mutations exhibit reduced cardiac function, mutants with C-terminal truncations also display disrupted skeletal muscle sarcomeres and paralysis. An additional promoter was identified within an intron of *ttnb* which produces a short *ttnb* transcript encoding only the C-terminal of the protein, and which is expressed at higher levels in skeletal muscle than cardiac muscle. Since this shorter C-terminal transcript is still expressed in embryos with N-terminal truncations of full-length

ttnb, this could explain why N-terminal *ttnb* mutations predominantly result in cardiac defects, while C-terminal mutations, which affect both transcripts, result in cardiac and skeletal defects. This internal promoter is conserved in mouse, and moreover the position of this putative promoter region lies at the border between alleles causing different phenotypic severity in the human *TTN* gene [34], providing insights into why the position of genetic lesions in *TTN* may contribute to DCM severity.

BLC2-associated athanogene 3 (BAG3) encodes a heat shock protein co-chaperone implicated in DCM. Initially, morpholino-mediated knockdown of zebrafish *bag3* was performed after *BAG3* CNV identification in a DCM [35], resulting in pericardial oedema and reduced fractional shortening in embryos, confirming a requirement for Bag3 in heart function. However, generation of a stable zebrafish *bag3* mutant revealed that while dispensable for embryonic heart function, *bag3* is required in adulthood. This is exemplified with adult *bag3* zebrafish mutants exhibiting a decline in cardiac function and reduced exercise tolerance at 6 months [36], consistent with DCM onset age in patients with pathogenic *BAG3* variants. Moreover, functional analyses of single myofibrils, isolated from dissected *bag3* mutant zebrafish hearts, revealed defects suggestive of hypocontractility, a phenotype associated with DCM rather than HCM, allowing classification of the cardiomyopathy in *bag3* mutants similar to that employed in patients. Comparative analysis of combinatorial *bag3*/*mTor* zebrafish mutants revealed that reduction of mTor signalling can partially rescue the hallmarks of DCM observed in *bag3* mutants [36]. This suggests that mTor represents a candidate therapeutic target for *bag3*-associated DCM, and that the *bag3* mutant may represent a suitable model to screen novel compounds to alleviate DCM associated with *BAG3* mutations.

The Laminin/Integrin/Integrin Linked Kinase (ILK) axis links the extracellular matrix to the cytoskeleton. The identification of a zebrafish *ilk* mutant, exhibiting hallmarks of DCM, provided the first evidence that disruptions to Laminin/Integrin signalling may result in cardiomyopathy [37]. Targeted sequencing of *LAMA4* and *ILK* in DCM cohorts subsequently revealed a missense mutation in *ILK* and 10 variants in *LAMA4* that were linked with DCM, representing the first identification of Laminin/Integrin mutations associated with DCM [37].
Heterozygous mutations in *ILK* have also recently been linked with ACM [38]. In this context, generation of transgenic zebrafish expressing either wild type or disease variant human ILK-GFP fusions demonstrated that these ILK variants cause fractional shortening at 3dpf, and specific variants result in death at juvenile stages [38]. Another zebrafish *ilk* mutant allele, *main squeeze (msq)*, carries a mutation in the kinase domain of ILK, and while DCM has not been described in *msq* mutants, they do have defects in cardiac contractility [39]. Protein Kinase B (PKB) phosphorylation is downregulated in *msq* mutants. However, injection of constitutively active PKB can restore heart contractility, suggesting that PKB phosphorylation is required for heart development. A subsequent study took advantage of this by using the *msq* mutants as a tool to screen for compounds which could restore contractile function [40], identifying

Calyculin A and Okadaic Acid as agents which could restore ventricular fractional shortening in *msq* mutants, as well as partially restoring PKB phosphorylation.

The ability to perform targeted knockdown, create disease-specific mutations, or overexpress disease variants have made zebrafish an invaluable model to validate novel candidate genes implicated in DCM, HCM and ACM. In addition to the better-characterised genes or pathways described above, functional studies in zebrafish have also identified novel roles for a diverse array of genes in cardiomyopathy e.g. *Raf1*, *Taf1a*, *Asn* [41-43]. Zebrafish DCM models have also been generated through pharmacological induction by administering the potassium channel blocker terfenadine to embryos [44], while a zebrafish model of ACM was developed using cardiomyocyte-driven expression of a plakoglobin disease variant, resulting in cardiomegaly and thinning of atrial and ventricular walls in early-adult fish [45] . By combining this ACM model with an *nppa*:luciferase transgenic line (upregulated in ACM zebrafish hearts), a chemical library was screened for compounds which would modify the upregulation of *nppa* in the ACM model, along with potential rescue of cardiac function. This approach identified that the compound SB216763, a GSK-3 inhibitor, could normalise defects in action potential observed in the ACM model [45], highlighting the tractability of the zebrafish model for performing functional screens for potential therapeutics which can alleviate DCM-type phenotypes.

Conduction disorders encompass a variety of pathologies, the precise nature of which depends on how generation or propagation of electrical impulses throughout the heart is disrupted. Despite the relative structural simplicity of the zebrafish heart compared to that of mammals, ECG analysis of adult zebrafish reveals that action potential dynamics of adult zebrafish cardiomyocytes is highly similar to human (Figure 3) [4-6]. Recent improvements to ECG in zebrafish include refinements in temperature considerations and the anaesthesia protocol to minimise impacts of environmental factors on action potential analysis [5, 46], optimisation of probe positioning and opening of the pericardial sac to improve signal-to-noise ratio [46]. These efforts to standardise measurements, and establish baseline variation in ECG parameters, allow more detailed analysis of how arrhythmia develops in the aging animal, in genetic models of arrhythmia, or upon inhibition of the parasympathetic nervous system [46]. Cardiac contractility in embryos and adults can be captured and quantified via echocardiography [47-49], while cardiac performance is also indirectly measured through exercise tolerance using swim tunnels, where adult zebrafish undergo timed swims against a current with increasing flow rate, and time to exhaustion is recorded.

In addition to the similarities between zebrafish and human cardiomyocyte action potentials, conduction dynamics in the zebrafish heart are also sensitive to channel blockers and activators used in humans, along with stimulants such as isoproterenol and norepinephrine [4, 5, 46, 47, 50-52]. Many of these compounds have similar effects on heart function or conduction as those observed in humans. Administration of compounds which stimulate sympathetic regulation of cardiac function, such as norepinephrine or isoproterenol, causes tachycardia in

zebrafish[5, 47, 50], while atropine, which inhibits parasympathetic input, also increases heart rate[46]. Ion channel modulators have similar impacts on cardiac conduction in zebrafish as in humans. ECG analysis of embryos exposed to compounds affecting potassium channels reveals a conserved atrial-specific response to acetylcholine/carbachol[4], while administration of E4031, stemizole, terfenadine, haloperidol, diphenhydramine, and orphenadrine (*hERG/KCNH2* K+ channel blockers) prolongs action potential duration (APD) and QT interval[4 50 7]. Analysis of action potential dynamics reveals that the sodium channel blocker tetrodotoxin reduces AP upstroke[4], while the L-type Ca channel blocker nifedipine shortened plateau phase, shaping AP duration[4]. Both nifedipine and the calcium channel antagonist verapamil induces bradycardia[5, 52] while BayK8644 (an L-type calcium channel activator) prolongs QT interval [50 52]. In some cases, these compounds also induce additional cardiac defects in a concentration-dependent manner, including arrythmias and AV block[7]. Conserved effects of anti-arrhythmic drugs also been demonstrated in zebrafish, with both amiodarone and quinidine (which can result bradycardia and prolong QT interval) similarly prolong QT interval in zebrafish[53 5 50]. However, not all compounds used in patients elicit the same response. HMR1556 (an I_{KS} blocker) shortened APD instead of prolonging it[4], while QT-prolonging drugs sotalol, erythromycin, quinidine, and amitriptyline did not have similar effect in zebrafish[52]. These discrepancies could result from some differences in ion channel composition or function, or poor uptake of the compounds. The impact of these pharmacological compounds on heart function can reveal information about the molecular pathways underlying cardiac electrophysiology in health and disease. This is enhanced by additional imaging tools, such as genetically-encoded calcium sensors driven by myocardial promoters which enable live *in vivo* imaging of transient calcium dynamics in the embryonic and larval heart [54-57]. Optical voltage mapping also provides high-resolution electrophysiological analysis of the heart. This can be achieved either through staining with a dye [58, 59], genetically encoded voltage sensors [55], or a recently-developed myocardial mitochondrial ATP sensor [60].

Long QT syndrome (LQTS), atrial fibrillation (AF), and sick sinus syndrome (SSS) are conduction defects that were modelled in zebrafish. LQTS is characterized by prolonged myocardial repolarization time, diagnosed by increased QT interval duration. Mutations in the ion channel *KCNH2* are causative for LQTS, and the zebrafish *breakdance* mutant, harbouring a *kcnh2* mutation, exhibits an atrioventricular (AV) block recapitulating that observed in paediatric LQTS [52]. The *breakdance* mutant was used as the background for a chemical screen for compounds which could suppress the AV block [61]. Administration of either 2-MMB or the steroid flurandrenolide shortened action potential duration (APD) and rescued AV block in the *breakdance* mutant, identifying two potential therapeutics which can shorten myocardial repolarisation. Patch clamp analysis, following the application of the I_{Kr} potassium channel blocker terfenadine to explanted hearts, reveals similarly increased AP duration to *kcnh2* mutants, providing further functional evidence that I_{Kr} is critical for repolarisation in the zebrafish ventricle [62].

Despite the fact that mutations in *KCNH2* are associated with LQTS, a heterozygous variant of *KCNH2* was associated with short QT syndrome (SQTS). Expression of this *KCNH2* variant in zebrafish revealed a gain-of-function phenotype [63], providing explanation for the opposing effect of the mutation on QT interval when compared to conventional LQTS-associated *KCNH2* mutations. The zebrafish *reggae* mutant provides further evidence that specific *KCNH2* variants can have opposing effects on QT interval. *reggae* mutants harbour a lesion in the voltage-sensing domain of *kcnh2* [64]. However, unlike the *breakdance* mutants, *reggae* mutants exhibit sinus block instead of AV block [52, 64]. Administration of terfenadine reduces potassium currents and rescues the phenotype of *reggae* mutants, suggesting the lesion results in gain-of-function. Supporting this, action potential duration is significantly reduced in *reggae* mutants, with a shorter QT interval [64]. Thus, *breakdance* and *reggae* mutants represent zebrafish models of LQTS and SQTS, with the *reggae* mutant representing the first described animal model for SQTS (Figure 3).

AF is characterised by irregular atrial pacing and often increased heart rate. Enhancer analyses in zebrafish identified putative *PITX2c* regulatory sequences which overlap genetic regions containing common AF-associated single nucleotide polymorphisms (SNPs) in humans suggesting dysregulation of PITX2 could be causative of AF[65]. ECG analysis of adult *pitx2c* zebrafish mutants reveals impaired cardiac function, accompanied by enlarged atria and increased atrial fibrosis [66]. This suggests *PITX2c* variants may be a causal factor in AF.

Sick Sinus Syndrome (SSS, also termed sinus node dysfunction) arises when the sinus node, the pacemaker of the heart, fails to generate a regular physiological heart rate resulting in arrhythmia. Mutations in *GNB5*, a guanine nucleotide-binding protein subunit involved in recruiting proteins to inward rectifier potassium channels, have been identified in individuals with early-onset sinus node dysfunction [67]. Analysis of the effect of carbachol and isoproterenol on a zebrafish *gnb5* mutant suggests that *GNB5* is required for parasympathetic control of heart rate, but not for sympathetic control. This indicates that loss of *GNB5* would be associated with extreme bradycardia at rest - a finding in line with abnormally low resting heart rates in patients with *GNB5* mutations [67]. Zebrafish studies validated gain–of- function activity of a *KCNJ3* variant identified in a family with autosomal dominant bradyarrhythmia [68], and demonstrated that administration of the I_{KACh} channel blocker NIP-151 improves bradyarrhythmia phenotypes in the zebrafish model.

Functional measurements, conduction sensors, and genetic mutants all provide suitable backgrounds that can be used as the foundation for pharmacological screens, identifying novel genes implicated in cardiac conduction, or compounds which modify conduction dynamics. A screen of almost 300 insertional mutants was carried out to identify new genes/pathways that regulate cardiac conduction. Cross-matching the positive hits with LQTS-associated GWAS data revealed a novel role for mitotic regulator *Gins3* in action potential

dynamics [58]. In another study, screening of synthetic compounds that restore heart function in *tremblor* mutants (a sodium-calcium exchanger *Ncx1* mutant with erratic heart contractility) revealed that Efsevin treatment can restore steady calcium transients and cell coupling, rescuing heart function [69].

Regeneration. The inability of human to replenish lost cardiomyocytes post-myocardial infarction (MI) results in persistent scarring, impaired heart function, cardiac remodelling, and eventually heart failure. Although the adult mouse heart has limited regenerative potential, neonatal mouse hearts are able to mount a regenerative response [70]. However, this capacity is lost by 7 days post-partum, and long term follow-up of neonatal mouse that have undergone ventricular resection suggests long term scarring as well as dilated cardiomyopathy [71]. Conversely, zebrafish are able to fully regenerate their hearts after injury, providing an invaluable model to understand the molecular mechanisms to improve regenerative potential in humans. Zebrafish models of heart regeneration have provided significant insights into the temporal processes underlying regeneration, along with the molecular mechanisms that underpin these responses. The early response from 3 hours post injury (hpi) includes expression of proinflammatory molecules and recruitment of immune [72-75]cells, which are important for scar deposition and the subsequent regenerative response. Simultaneously, endocardial cells in the uninjured tissue undergo morphological changes and re-express developmental genes [76]. After inflammation and endocardial activation have occurred, the endocardium and epicardium are the first cell layers to undergo large-scale regeneration. Between 3 to 5 dpi endocardial cells around the injury site proliferate [77], before migrating to cover the internal face of the wound area. Onset of coronary revascularisation is initiated rapidly post-injury, and the vascular network is required to promote regeneration[78, 79]. ECG analysis of regenerating hearts shows that despite prolongation of QT interval during regeneration, action potential dynamics return to normal post regeneration [53, 80].

Several zebrafish cardiac injury models have been established (Figure 4), with ventricular amputation capturing the ability of the heart to undergo regeneration, forming a fibrin clot which is gradually replaced with muscle 30-60 days after injury [81, 82]. Myocardial infarction (MI) induces death of multiple cell types alongside inflammation and fibrosis. Thus, regeneration requires not only the replacement of lost cardiomyocytes, but also the removal of dead cells, matrix remodelling, revascularisation, and re-establishment of electromechanical coupling in the heart [83]. This can be modelled in zebrafish by cryoinjury which results in substantial localised cell death in the injured portion of the ventricle, resulting in apoptosis of all cardiac cell types and recapitulating the cardiac necrosis that occurs post-MI [84]. Inducible genetic ablation models, where cell type-specific promoters drive expression of either diptheria toxin (DTA), or nitroreductase (Ntr, an enxyme which converts Metronidazole into a cytotoxic agent) provides further mechanistic insights into how different cell types contribute to cardiac regeneration [85-89].

Interestingly, while the regenerative response to cryoinjury is robust, scar resorption diminishes with repeated injuries and after 6 cryoinjuries hearts fail to resolve fibrotic tissue [90]. While this demonstrates that the heart can regenerate after multiple insults (the ability to regenerate cardiomyocytes themselves does not appear to be impacted after multiple injuries), it suggests there is a limit to the ability to replace the fibrotic tissue with new cardiomyocytes. The multiple-injury model provides opportunities to separate the pro-regenerative programme from that of fibrosis and scar resolution, which may have implications for improving cardiac function in MI despite the presence of scarring to the heart. The combination of zebrafish genetic and regeneration models can also provide insights into how cardiac dysfunction impacts regenerative capacity, and how therapeutics may be developed to improve this capacity. Cardiac regeneration is impaired in the zebrafish *breakdance* mutant LQTS model, associated with increased extracellular matrix (ECM) deposition and excessive inflammation[91]. While administration of inflammatory compounds such as dexamethasone or matrix metalloproteinase (MMP) inhibitors promotes scar resolution and regeneration in this model [91], timing and evolution of the immune response during regeneration is likely to be crucial in mediating regeneration[92], and zebrafish represent an excellent model to directly investigate how the immune response could be manipulated to promote regeneration in humans. Similarly, targeting ECM-remodeling represents a promising therapeutic avenue. A 2016 study demonstrated that ECM from regenerating zebrafish hearts has pro-regenerative effects in mammalian non-regenerative models[93] suggesting that specific ECM composition may be key in promoting specific aspects of regeneration. In line with this, recent studies have reported that administration of the ECM component Agrin to post-MI mouse and pig hearts improves cardiac regeneration[94, 95], demonstrating that insights from zebrafish can lay the foundations for developing therapeutic strategies.

While significant insights into cardiac regeneration have been made using zebrafish, one limitation lies in the ability to monitor morphological and functional recovery live, relying on fixed tissue analyses (although light-sheet imaging facilitates visualisation of regeneration within whole-tissue context). Advances in MRI imaging of the regenerating heart provides new opportunities to assess regeneration in the same animal over time [96], while the development of a fluidic device to culture explanted injured hearts allows live imaging of processes such as revascularisation, providing more detailed insights into specific cellular interactions during regeneration [97].

Together, the ability to perform live, in vivo analyses of cardiac development, function, and regeneration in zebrafish provides a unique opportunity to define the relationships between morphological and functional abnormalities during development, and cardiac dysfunction and structural remodelling over the life course.

VASCULAR DISEASE MODELS

Endothelial dysfunction and atherosclerosis.

Cardiovascular development is a complex and tightly regulated process, highly conserved among vertebrates. Many of the conserved developmental pathways regulating cardiovascular development, including BMP, TGFβ, Notch and Wnt, are re-activated in arteries in adults and play critical roles in the development of cardiovascular diseases, including atherosclerosis [98]. Thus using zebrafish embryos and larvae not only as a vertebrate developmental model, but as a model for processes involved in human diseases, may provide important insights into molecular mechanisms regulating vascular disease (Figure 5).

Atherosclerosis is a chronic inflammatory disease that can lead to myocardial infarction and stroke. Despite the systemic nature of many of the associated risk factors, atherosclerosis is a focal disease which develops in the regions of arterial trees where wall shear stress (WSS) magnitude is low and flow patterns are disturbed, this is particularly the case at vessel bends, branches and bifurcations. Disturbed flow and low WSS activate endothelial cells (ECs) leading to a pro-inflammatory, proliferative, and pro-apoptotic phenotype, contributing to endothelial injury and leading to development of atherosclerotic lesions. In contrast, laminar, unidirectional flow, and physiologically high WSS confer a protective, quiescent phenotype to ECs. Transparency of the zebrafish embryos combined with availability of transgenic lines allow imaging and measurement of blood flow parameters and haemodynamic forces in the developing zebrafish vasculature using digital particle image velocimetry (DPIV) and computational fluid dynamics (CFD). These studies indicate that WSS magnitudes in the zebrafish vasculature are comparable to those in humans, with Lee *et al*. reporting the mean WSS in the dorsal aorta between 4 and 8 dyne/cm2 and in the caudal vein between 1 and 2 dyne/cm2 at 15 days post fertilization [99]. In contrast, WSS magnitudes in mice are approximately ten times higher than in humans, primarily due to differences in size and heart rate. [100]

Although zebrafish have been used to study the roles of flow and WSS in vascular development and remodelling[101-107] endothelial responses to flow, relevant for development of atherosclerosis, require further examination. In a recently established zebrafish model of flow-mediated EC apoptosis, preventing blood circulation using *tnnt2a* morpholino (*silent heart* embryos) or the anaesthetic tricaine, resulted in increased EC apoptosis in the vascular plexus of embryos.[108] The model was subsequently used for functional validation of a number of putative apoptotic regulators found to be enriched at a disease prone site. It will be of interest to determine whether haemodynamic forces have an effect on other features of EC dysfunction in zebrafish, such as proliferation and inflammatory responses.

Reduction of viscosity-dependent shear stress in zebrafish embryos decreased induction of the glycolytic metabolite, dihydroxyacetone by flow-sensitive VEGFR-PKCε-PFKFB3 signalling. This led to impaired vascular regeneration following zebrafish tail amputation, while injecting erythropoietin mRNA to increase shear stress promoted vascular repair [109]. In conclusion, zebrafish embryos can model several features that are important in the pathogenesis of atherosclerosis including EC apoptosis, impaired vascular repair and regeneration.

Lipid metabolism.

In addition to haemodynamic factors, lipid metabolism plays an important role in atherosclerosis development. Accumulation of low density lipoprotein (LDL), the main carrier of cholesterol, in the subendothelial layer and its subsequent oxidation into oxidised LDL triggers inflammatory and immune responses, which initiate plaque formation. Interestingly, feeding zebrafish a high cholesterol diet (HCD) can replicate some of the processes involved in the early stages of atherosclerosis, such as hypercholesterolaemia, lipoprotein oxidation, vascular lipid accumulation, and myeloid cell recruitment to the vasculature [110]. Feeding of HCD to zebrafish for up to 10 days starting from 5 days post fertilization (dpf) resulted in increased expression of inflammatory markers TNFα and Il1β, as well as decreased expression of anti-inflammatory gene PPARγ in the endothelium, prior to myeloid cell accumulation and lipid deposition [111].

So far, two zebrafish genetic models of hyperlipidaemia have been reported: apolipoprotein C-II (*apoc2*) and LDL receptor (*ldlr*) mutants which have been generated using TALEN- and CRISPR/Cas9-mediated genome editing, respectively [112, 113]. In accordance with the phenotype observed in human patients with APOC2 deficiency, Apoc2 zebrafish mutants fed a normal diet displayed hypertriglyceridaemia, which was rescued by injection of plasma from wild-type zebrafish or by injection of a human APOC2 mimetic peptide. Apoc2 deficient zebrafish larvae exhibited accumulation of lipid and lipid-laden macrophages in the vasculature, in resemblance to early human and mouse atherosclerotic lesions[112]. *dlr* mutants, on the other hand, developed moderate hypercholesterolaemia when fed a normal diet, which was exacerbated following a short-term, 5 day HCD feeding starting at 4.5 dpf [113]. Therefore, hyperlipidemia can be modelled in zebrafish using either dietary and genetic approaches and such models can be useful for screening of novel lipid-lowering compounds.

Vascular ageing is a significant and independent risk factor for a range of degenerative diseases, including cardiovascular diseases. Vascular ageing is characterised by increased endothelial dysfunction and medial wall calcification, contributing to arterial stiffness [114]. Zebrafish are an attractive model for studying age-related diseases, due to their short life span, which is similar to that of mice, and conservation of mechanisms involved in DNA damage repair and regulation of telomere length. Interestingly, telomere shortening *in vivo* has been observed in the regions susceptible to atherosclerosis and telomere lengths are shorter in atherosclerotic plaques compared to healthy vessels [115], indicating a role for telomere homeostasis in the pathogenesis of atherosclerosis.

Neurovascular physiology and stroke.

Due to the combination of larval transparency, experimental accessibility of embryos, and experimental tools available, zebrafish have become a crucial model to study neurovascular development. This is exemplified by the ability to study blood-brain-barrier (BBB) formation *in vivo* and over time [116-120], showing that BBB formation and cerebrovascular angiogenesis occur in parallel [121] and the identification of functional and genetic regulators for BBB formation [122-124]. Recent work showed that a functional interaction between the brain vasculature and neurons (i.e. neurovascular coupling, NVC) develops in zebrafish between 6-to-8 dpf [125], establishing the first non-rodent model to study NVC. Similarly, careful

observations revealed the existence of perivascular cells [126], shed light on vascular mural cell coverage [127, 128], established the dynamics of cerebrovascular spinal fluid movements [129], and discovered a new endothelial cell membrane behaviour, termed *kugeln* [130]. Increasing understanding in vascular diseases and the ability to model those in zebrafish, such as stroke [131]or thrombosis [132], sheds light on vascular bed specific disease progression, such as vascular dementia. Additionally, we are beginning to understand how vascular regulation develops[133]. This is complemented by studies examining the role of blood flow on cerebrovascular patterning and cell death, as well as novel computational analysis approaches which bridge the gap between data acquisition and extraction of meaningful results for clinical translation using quantitative objective image analysis [134].

Ischaemic stroke, which results from occlusion of cerebral blood vessels, accounts for ~85% of all acute strokes. On the other hand, haemorrhagic stroke, caused by intracerebral haemorrhage, is less common (~15%) but it is associated with higher morbidity and mortality rates. There have been a limited number of studies attempting to establish a zebrafish model for studying ischaemic stroke[135]. In contrast, zebrafish have more extensively been used to study intracerebral haemorrhage; it has recently been demonstrated that bleeding in the brain of zebrafish larvae induces quantifiable pathological and inflammatory phenotypes which mimic key features of human intracerebral haemorrhage[136]. One of the advantages of zebrafish over mammalian models is the ability of zebrafish larvae to exhibit spontaneous brain-specific bleeding which can be observed noninvasively. Intracerebral haemorrhage can result from genetic mutations (*arhgef7*, *pak2a*, *notch3*) or can be induced pharmacologically (atorvastatin)[137, 138]. However, there are limitations to using the zebrafish model which include the lack of fully developed cranium and rapid recovery rates from brain injury[136].

CARDIOVASCULAR DRUG DISCOVERY AND FUTURE DIRECTIONS.

Despite considerable advances in therapeutic approaches, cardiovascular diseases are still the leading cause of death worldwide. The zebrafish model offers exciting possibilities for drug discovery by combining advantages of both in vitro systems (including cost, size, ease of use and amenability to automation) and mammalian systems, as a holistic and physiologically relevant model with high level of genetic and functional conservation between zebrafish and humans[139].

However, limitations of the zebrafish model must also be considered. Firstly, high throughput screening is limited to water-soluble compounds that can be added to the water in which the fish develop. Additionally, due to solubility issues, especially at higher compound concentrations, it may be challenging to predict the relationship between administered compound dose and actual exposure. While methods for robotic injection into the zebrafish yolk are available and suitable for injection of DNA, microbes or cells [140], microinjection into blood circulation is less amenable for high throughput applications. Secondly, lack of standardized protocols, including zebrafish strains, husbandry practices and experimental design, can lead to experimental variability and lack of reproducibility [141]. Despite these

limitations, there are currently eight compounds discovered in zebrafish that have advanced into clinical trials which demonstrates the translational value of the zebrafish model [142]. The combination of genetic and pharmacological models mimicking human cardiac dysfunction (including advances in genome-editing which allow the creation of human disease-specific variants in zebrafish), high resolution live in vivo imaging, electrophysiological analysis of cardiac function, and high-throughput screening capacity, position the zebrafish as an excellent translational model. This is exemplified by screens identifying compounds which improve cardiac function in specific zebrafish cardiomyopathy models[40, 45 61, 68, 69], providing the foundation further investigation.

Recent technological advancements allow for fully automated high throughput screening assays, with robotics-mediated handling steps and automated image acquisition and analysis. Burns et al established a 96-well-based assay for measuring heart rate using automated microscopy of *Tg(cmlc2:GFP)* embryos expressing GFP specifically in the myocardium [143]. Some of the challenges, such as the requirement for anesthaesia/restraint, fluorescently-labelled animals, as well as the level of throughput, have been addressed in subsequent studies [144, 145]. A wide range of cardiovascular parameters, including heart rate, rhythm, contractility, vessel diameter and blood circulation, can now be assessed automatically or semi-automatically using 96-well or 384-well format [146].

In addition to drug discovery, zebrafish is also a valuable model for cardiotoxicity studies. Cardiotoxicity, manifested by conduction abnormalities, arrhythmias and depression of myocardial contractility is a serious side effect of many drugs which can limit their clinical application. One small molecule screen in zebrafish found that 22 out of 23 tested compounds which cause QT interval prolongation in humans, caused bradycardia and atrioventricular block in the zebrafish, demonstrating suitability of the zebrafish model [147]. An example of a cardiotoxic agent is doxorubicin, a highly effective chemotherapy drug, the use of which is limited by cumulative, dose-related myocardial damage which can lead to heart failure. Using a zebrafish model of doxorubicin-induced cardiomyopathy for small-molecule screening, cytochrome P450 family 1 (CYP1) has been recently identified as a candidate therapeutic target for clinical cardioprotection [148, 149].

Zebrafish, first introduced as a developmental model in 1970s, have become an increasingly attractive and valuable model for studying human diseases. With its unique advantages, the zebrafish model has proven its utility in studying disease mechanisms, drug screenings and toxicology studies, complementing murine models and *in vitro* systems. With the emergence of large amounts of data generated by human –omics studies, identification of new, robust and reliable zebrafish models for functional screening of genes with putative roles in cardiovascular health and disease is required. Zebrafish are emerging as a valuable model for functional validation of GWAS data from patients with diverse cardiovascular disease, providing additional disease-specific models which can inform therapeutic development[150].

Despite its amenability to high throughput screening, automated imaging and phenotypic scoring still remain a challenge. Continued technological advances and application and

development of automated zebrafish screening platforms will further clinical significance of the zebrafish model, leading to better understanding of disease mechanisms and new therapeutic targets for human diseases, including cardiovascular disease.

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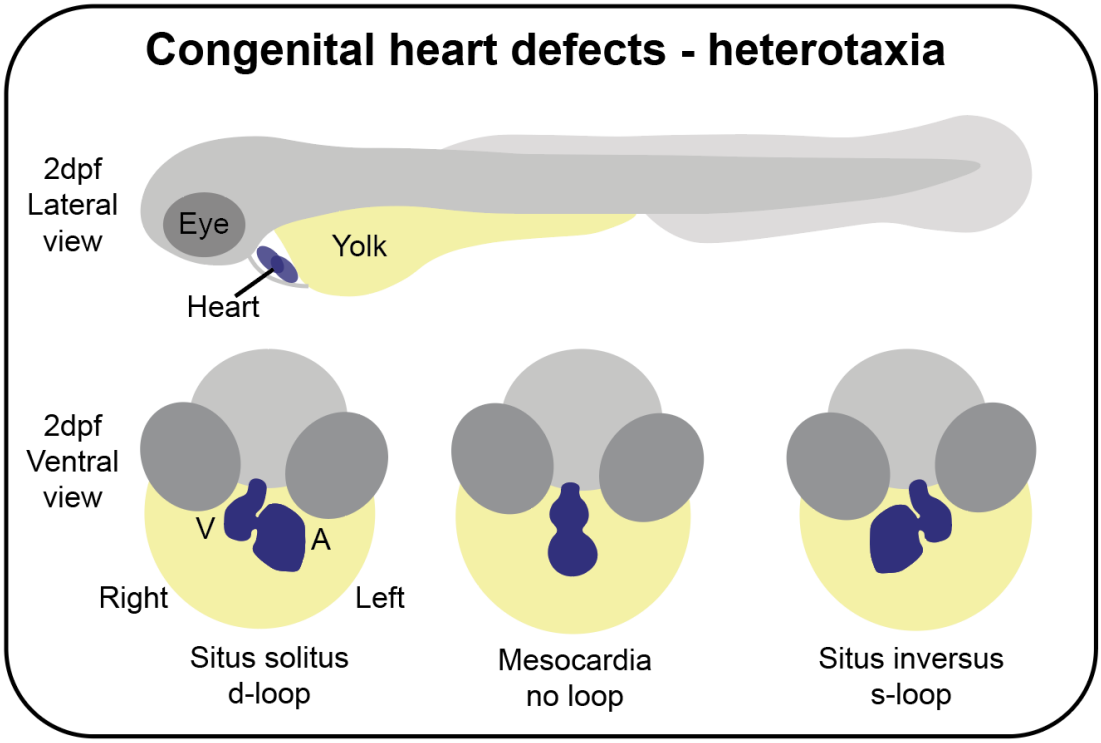

Figure 1 – Zebrafish models of congenital heart defects.

Congenital heart defects in zebrafish are often analysed at 2dpf, when the heart has undergone initial looping morphogenesis and is positioned over the yolk, ventrally and posterior to the head. Morphology of the atrium (A) and ventricle (V) can be distinguished, and the atrioventricular canal and outflow tract can be visualised. Heterotaxia phenotypes can be assessed by the directionality of looping morphology of the heart, which usually undergoes a d-loop under normal left-right patterning (situs solitus), but can be reversed (s-loop) or remain at the midline (no-loop) if embryonic laterality is disrupted.

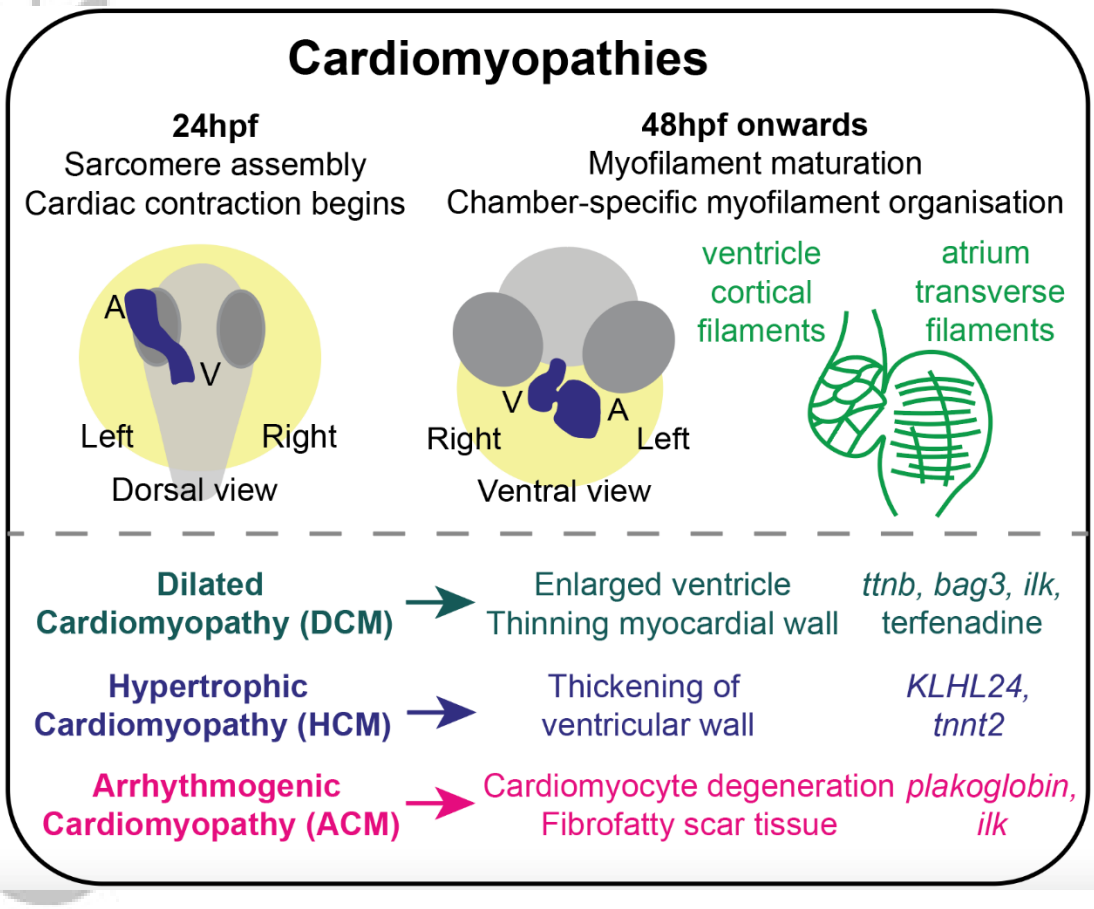

Figure 2 – Zebrafish models of cardiomyopathy.

Cardiac contractility in zebrafish begins around 24hpf, when the heart tube has first formed and sarcomeres are assembled. As the heart develops the myofilaments mature, and by 48hpf atrial and ventricular cardiomyocytes display different myofilament organisation. Ventricular wall cardiomyocytes displaying cortical basal actin while atrial cardiomyocytes forming long myofilaments spanning the cell running perpendicular to the direction of blood flow. Disruptions in sarcomere assembly and function result in diverse cardiomyopathies, represented by zebrafish mutants or misexpression models, including those listed.

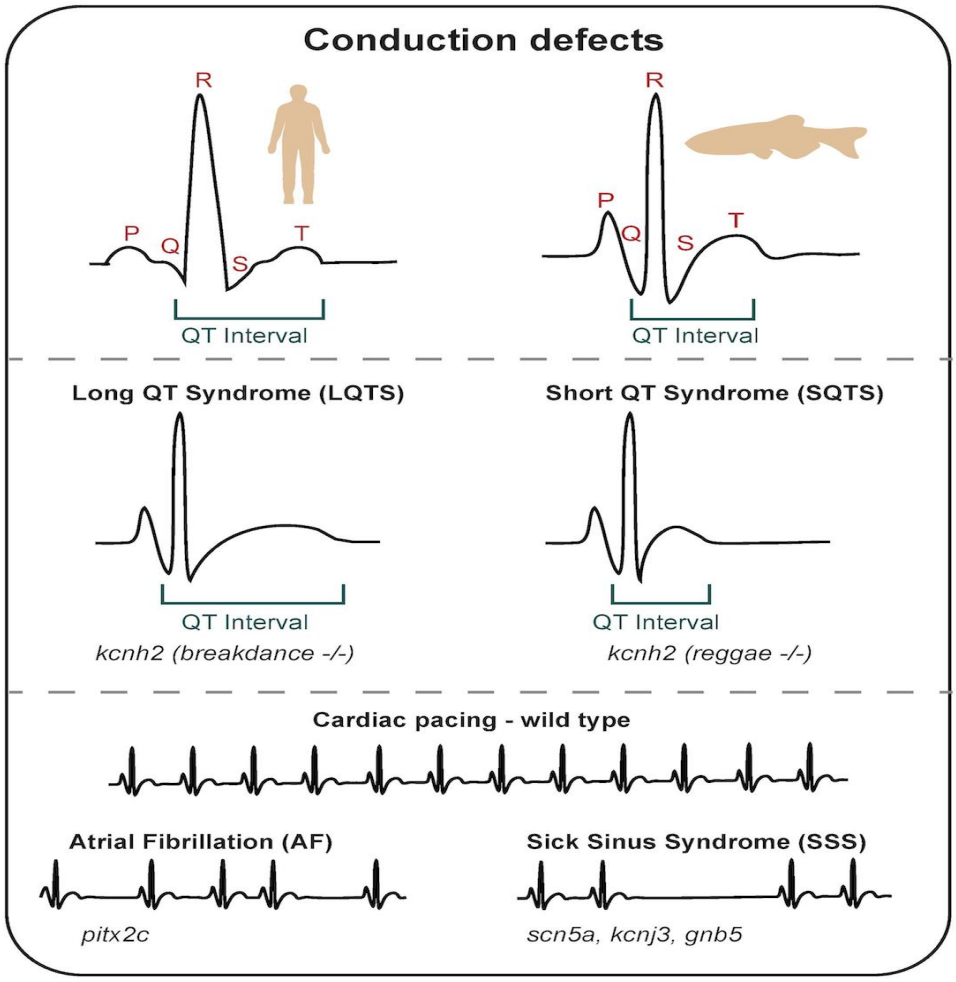

Figure 3 – Zebrafish models of cardiac conduction defects.

ECG recordings of embryonic and adult zebrafish hearts closely resemble those obtained from humans, with distinguishable P wave, QRS complex, and T wave, allowing quantification of comparative parameters such as QT interval. Both Long and Short QT Syndrome (LQTS and SQTS) can result from mutations in the potassium channel *kcnh2*, and are modelled by the zebrafish *breakdance* and *reggae* mutants respectively. Cardiac pacing defects such as Atrial Fibrillation (AF, loss of regular sinus rhythm) and Sick Sinus Syndrome (SSS, defective sinus pacing, including sinus pause) have also been modelled using zebrafish loss of function and misexpression models.

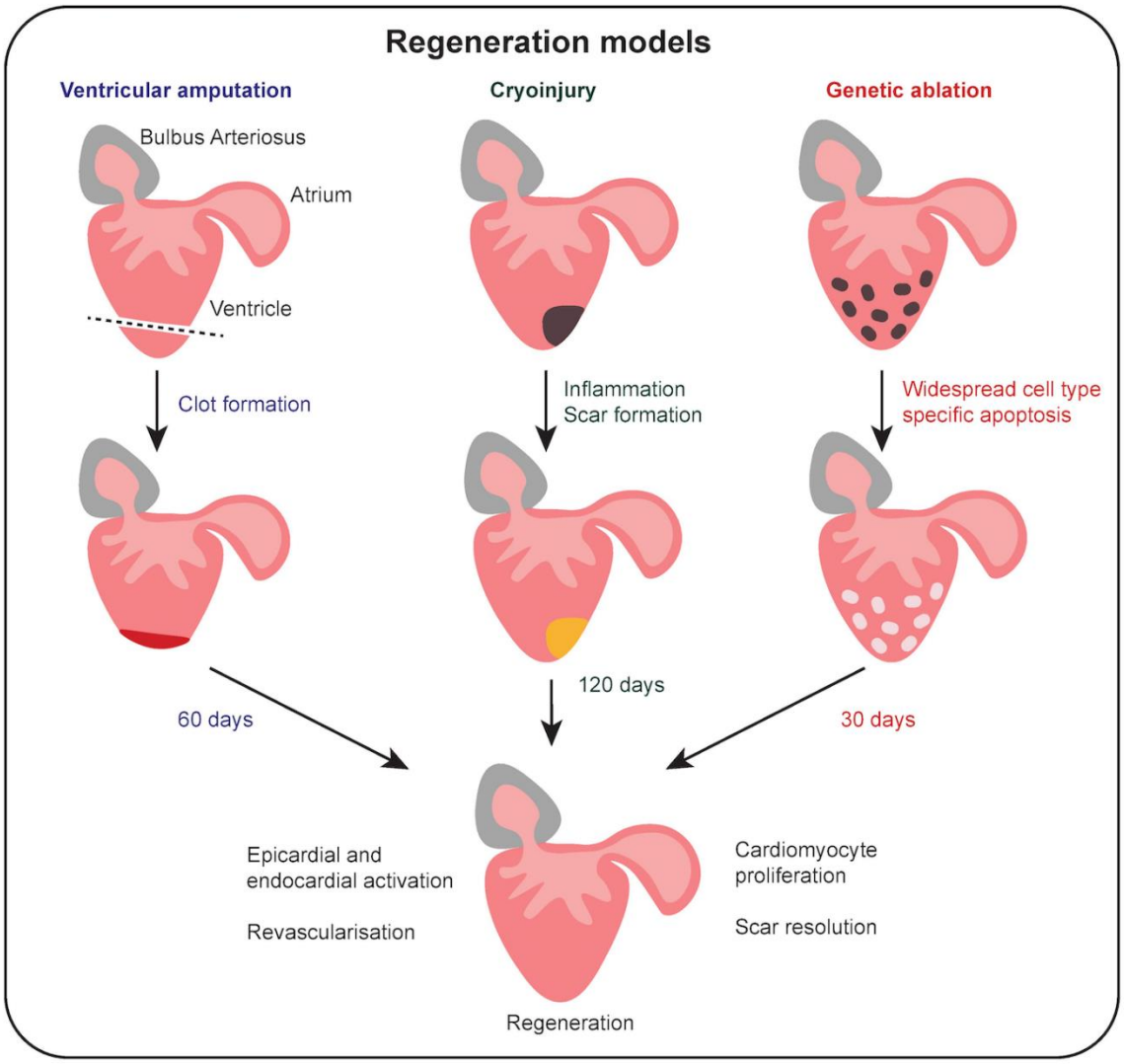

Figure 4 – Zebrafish models of regeneration.

The zebrafish heart is capable of fully regenerating after injury. In the resection model the ventricular apex is amputated resulting in formation of a fibrin clot, and new heart tissue grows within around 60 days. In the cryoinjury model a cryoprobe is applied to the ventricle causing localised cell death. Inflammation and clearance of cell debris subsequently occur, and a scar is formed at the injury site. This scar is resolved after around 120 days, and the heart is regenerated.

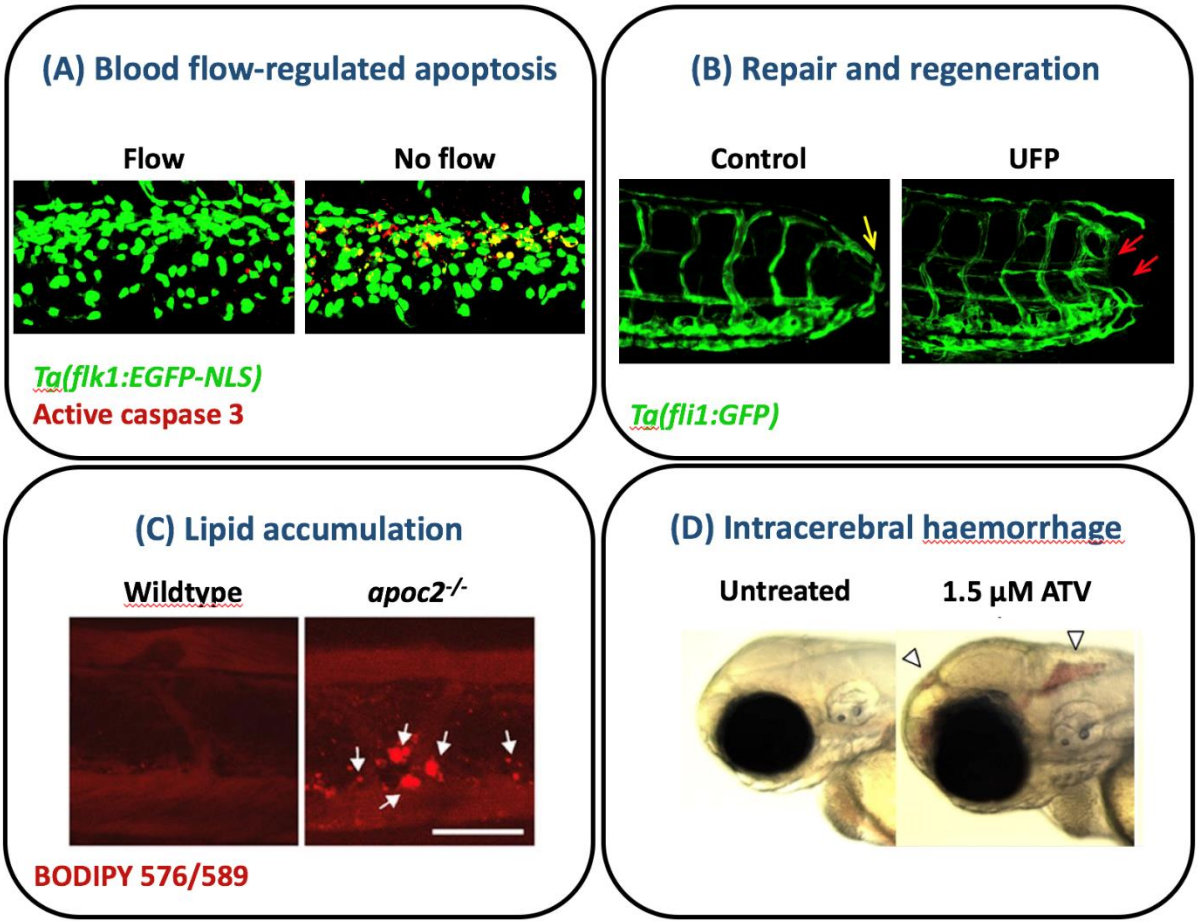

Figure 5 – Zebrafish embryos and larvae can be used to study vascular responses to flow, high fat feeding and as models of intracerebral haemorrhage. (A) Blocking blood flow leads to increased endothelial cell apoptosis in the aorta and caudal vein plexus of zebrafish embryos at 30 hours post fertilisation (hpf). Whole-mount active caspase-3 (red) staining of 30 hpf *flk1:EGFP-NLS* zebrafish embryos (green EC nuclei) in the presence or absence of flow (*sih* morpholino oligonucleotide). Apoptotic ECs are shown in yellow. (B) Vascular injury by tail amputation at 3 days post fertilization (dpf) results in shear stress-dependent vessel repair and regeneration by 3 days post amputation (dpa), which is impaired upon exposure to ultrafine particles (UFP). Control fish developed vascular regeneration connecting the dorsal aorta with the dorsal longitudinal anastomotic vessel at 3 dpa (yellow arrow). Fish exposed to UFP developed impaired vascular repair and a disrupted vascular network (red arrows) at 3 dpa. (C) Vascular lipid deposits in 14 dpf wildtype (WT) and *apoc2* mutant larvae (*apoc2*$^{-/-}$) fed a normal diet. (D) Exposure of zebrafish embryos to atorvastatin (ATV) results in intracranial haemorrhage. Bleeds formed in both forebrain and mid-hindbrain regions (arrows denote haemorrhages). (B) Images kindly provided by Prof. Tzung Hsiai. Images adapted from Liu et al 2015 (C) and Crilly et al 2018 (D) under Creative Commons License.