Interrogating Cardiovascular Genetics in Zebrafish

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Emerged as a highly versatile and applicable vertebrate animal model to study embryonic development, zebrafish over the last two decades became a valuable human disease model for cardiovascular research. The unprecedented in vivo imaging capabilities allowing live analysis of organ formation and organ function combined with the ease of precise genetic interrogation established zebrafish as a recognized cardiovascular disease model. This chapter provides an overview of

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zebrafish's history in biomedical and particularly cardiovascular research, delivers an outline of available methodologies and resources when using zebrafish, and gives examples of the versatility of zebrafish in cardiovascular research and how it progressed our understanding of a given disease.

9.1 From a Developmental Model to a Disease Model

9.1.1 The Beginnings and the Rise in Popularity

In the 1980s, Dr. George Streisinger and his colleagues at the University of Oregon laid the groundwork for using the zebrafish as a genetic model. He focused initially his pioneering work on developmental genetics based on several practical advantages and genetic amenities. The idea of having a vertebrate model with which genome-wide forward genetic screens are feasible as performed before in worms, flies, and plants to uncover novel gene functions ultimately materialized in 1989 was carried out by a colleague of Streisinger at the University of Oregon [1]. Inspired by the success and the potential of this first forward genetic screen, scientists from two groups in Boston and Tübingen conducted the first large-scale forward genetic screen in a vertebrate model. This screen recovered over 800 mutants with mutations in 372 genes uniquely involved in the development of the central nervous system, intestines, blood, bone, vasculature, and the heart to only name a few [2, 3]. Even over 20 years after conducting this screen, zebrafish mutants from it are still being used and analyzed in labs worldwide, illustrating the huge impact and the value this screen had and still has in the community.

Zebrafish are approximately 3–5-cm-long fish belonging to the *teleost* family with inexpensive housing and modest space requirements. When mated, a single female can produce between 100 and 300 eggs per week. Sexual maturity is reached with the age of 3 months. Particularly tempting as a developmental model is its extrauterine development and its fast ontogeny. Its small larval size of 1–2 mm allows analysis throughout the entire development, while macroscopic cell experiments like cell transplantations remain feasible and efficient. Gastrulation begins at around 5 h postfertilization (hpf) and segmentation, and with that somitogenesis, by 10 hpf (Table 9.1) [4]. Embryonic vasculogenesis is completed by 18 hpf, and angiogenesis can be observed before 20 hpf, and the first primitive heart tube initiates first contractions within the first day of development. Already 3 days postfertilization (dpf), the cardiovascular system of the zebrafish is comparable to that of a newborn mammal and reaches full maturity by 5 dpf, in a by-then organism exhibiting most of the internal organs found in humans [5]. In contrast to mammalian models, zebrafish does not rely on a functioning cardiovascular system during the first 5 days of life, since oxygen and nutrient supply is achieved exclusively through passive diffusion. Even substantial cardiovascular deficiencies can thus be tolerated and fish, even without any observable heart activity or blood flow, develop overly normal without systemic compromising impact due to secondary adverse effects within the first 5 dpf [6].

Table 9.1 Comparison of timepoints when key cardiovascular developmental events happen in zebrafish, mouse, and humans

	Zebrafish	Mouse	Human
Gastrulation	5 hpf	E6.5	17 days
Somitogenesis	10 hpf	E7–E7.5	17–18 days
Cardiac progenitors migrate and fuse at the midline	12–18 hpf	E8	19 days
First contractions of the primitive heart tube	21–22 hpf	E8.5	22 days (3 weeks)
Heart chambers visible	30 hpf	E8.5	22 days (3 weeks)
Looping	30–36 hpf	E8.5	22 days (3 weeks)
Valve formation	45 hpf	E9	30 days
Ventricular septation	–	E15	90 days
Atrial septation	–	After birth (day 21)	After birth (appr. 40 weeks)
Cardiovascular maturity	5 dpf	4 weeks	8–12 years
Vasculogenesis-primitive plexus formation	20 hpf	E8–8.5	1st trimester (up to week 11)

E embryonic day

9.1.2 The Uprising Disease Model

Not only because of these obvious advantages but also with the 1996 mutagenesis screens and the recovery of mutant alleles modeling known human diseases, the zebrafish began its journey toward challenging the till-then dominating mouse model in recapitulating human disease conditions. As a nonmammalian model though, the zebrafish comes with apparent limitation, the most obvious being that zebrafish lack some of the organs found in humans, like lungs and mammary gland. The first zebrafish mutant reported modeling a human pathology and contributed to a better understanding of the underlying pathomechanism was *yquem* (*ype*) [7]. Zebrafish and humans share remarkable conservation in hematopoiesis, and *ype* presented a photosensitive porphyria syndrome as seen in patients. Although porphyria in humans was already associated to a gene encoding uroporphyrinogen decarboxylase (UROD), the study of *ype* now explained the exact enzymatic defect leading to the disease also in humans [7].

Subsequently, publications on zebrafish modeling various human pathologies dramatically increased. While modeling blood disorders in zebrafish dominated initially, zebrafish models for human diseases ranging from ocular disorders and neurodegenerative diseases to muscular dystrophies, cancer, addiction, behavioral disorders, and cardiovascular diseases have been described [8–10]. In particular, concerning congenital heart malformations, like valve deficiencies, to aneurisms, cardiomyopathies, and arrhythmias, zebrafish is now a well-established cardiovascular disease model. Although having a two-chambered heart instead of a four-chambered heart as in mammals, the zebrafish was proved to be particularly valuable

for cardiac electropathophysiology (see section below) as compared to mice, since zebrafish action potential shape, heart rate, and the electrocardiogram closely resemble that of humans [11].

9.2   Genetics and Genetic Interrogation in Zebrafish

The ease of performing even large-scale genetic studies in zebrafish, which in the past also contributed significantly to a more detailed understanding of genetics in human disease, makes a high-quality genome sequence and complete annotation essential. The zebrafish is now further frequently used as an independent functional validation tool for findings from human genetics studies [12]. A recent study, initiated in 2001 by the Wellcome Sanger Trust Institute, identified and annotated 26,000 individual genes encoded on the zebrafish's 25 chromosomes compared to the roughly 20,500 genes in the human genome [13]. Sequencing additionally revealed that teleosts underwent a whole-genome duplication, with 25–30% of the duplicated genes still retained in zebrafish [13, 14]. Thus, frequently duplicated genes are found when screening for human disease gene orthologues, adding potentially to a functional validation experimentation since duplicated genes often exhibit subfunctionalization properties. Seventy percent of the human genes have a direct homologue in the zebrafish. More astounding is that 84% of the known human disease genes have a direct homologue in fish [13].

Along with this solid foundation, the zebrafish comes with a highly versatile tool set to interrogate gene functions and identify genes with so far unappreciated functions. As traditionally introduced and envisioned by its pioneers, the zebrafish was and is a powerful forward genetics model.

9.2.1   Forward Genetics

Classical chemical mutagenesis screens, in particular deploying *N*-ethyl-*N*-nitrosourea (ENU), are easy and unprecedented in zebrafish compared to any other vertebrate model and enable production of thousands of mutants in a relatively short time (reviewed here [15]). While being easy and producing mutations very efficiently with little genomic bias, chemical mutagenesis screens have the disadvantage that subsequent identification of the gene mutation causative for the observed phenotype is time- and resource-consuming. To overcome this limitation, insertional mutagenesis techniques using replication-deficient pseudotyped retroviruses or transposons were introduced by several groups [16–18]. In addition, and initially adapted from mouse, the *Sleeping Beauty* DNA transposon somatic mutagenesis system was successfully introduced in zebrafish and led to the identification of conserved and novel human cancer genes in zebrafish [19]. Insertional mutagenesis, due to its predesigned insertional cassettes, including reporters, enhancers, silencers, and amplification tags, allows rapid identification of the targeted gene. However, this comes with the price that retroviruses and transposons typically achieve a very much

lower mutagenic efficiency than ENU, requiring considerably larger libraries of mutagenized fish.

Using sensitized genetic backgrounds to perform modifier screens is a powerful way to isolate genetic interactions of genes. Since screening of several genomes and large libraries of mutagenized animals is necessary, this was longtime reserved for invertebrate models, including *Drosophila* and *C. elegans*. Kramer et al. performed the first enhancer screen for modifiers affecting dorsoventral patterning by using heterozygous zebrafish mutants for the *chordin* gene and identified initially seven enhancer mutations [20]. Bai et al. screened for genetic suppressors of a mutation in the *moonshine* (*mon*) gene, which encodes for the transcriptional intermediary factor 1 gamma (*tf1γ*) [21]. *mon* mutants exhibit anemia and larval lethality. They utilized a complex haploid screening strategy and identified the *sunrise* mutation that fully normalized globin content in *mon* mutants. Interestingly, sunrise encodes for cdc73, a component of the Pol II transcriptional elongation complex, for the first time linking *tf1γ* function and transcriptional elongation to erythroid development.

9.2.2 Reverse Genetics

The so far numerous forward screens performed in zebrafish to date, despite the significant time, staff, and resource efforts, have led to the identification of plentiful new genes and conserved pathways involved in human disease, including cardiovascular disease [22–24]. However, not only because zebrafish became a valuable model to functionally validate candidate genes but also because of advances in sequencing and other large-scale gene identification technologies, including high-throughput automated gene expression analysis, i.e., in situ hybridization screenings, reverse genetic tools to interrogate the function of distinct genes have been established [25, 26]. Development of available technologies adapted to zebrafish research accelerated dramatically during recent years (Table 9.2).

First reverse genetics in zebrafish was performed based on technologies that were well established and combined with advanced sequencing leading to targeting induced local lesions in genomes, or TILLING for short [28]. In TILLING, libraries of ENU-mutagenized F1 families are sequenced and sperm cryopreserved. Sperm can be revitalized by in vitro fertilization when a locus of interest is affected by an induced mutation. Hundreds and thousands of cryopreserved sperm from TILLING efforts are stored in huge facilities and catalogued. They are available to the research community through the Zebrafish International Research Center (ZIRC, www.zebrafish.org) at the University of Oregon, USA, and the European Zebrafish Research Center (EZRC, http://www.ezrc.kit.edu) at the Karlsruhe Institute for Technology (KIT), Germany [29].

With the introduction of morpholino-modified antisense oligonucleotides (MO) adapted from *Xenopus,* an easy, fast, efficient, and specific gene silencing tool when well controlled [30] to analyze the function of a distinct gene became available [31]. MOs do not target the genomic locus of a gene but rather bind to the transcript to cause aberrant translation or splicing. They do so either by targeting the

Table 9.2 Overview of available reverse genetics tools

Reverse genetic tools	Mechanism	Pros	Cons	Resources
Morpholino-mediated gene knockdown	Synthesized oligonucleotide analogs disrupt splicing or attenuate translation	Fast, convenient, modified caged MO can be used for conditional gene knockdown, rapid identification of affected gene	Not inheritable, effective within first 3–5 days of development, prone to off-target effect	Gene tools, http://www.gene-tools.com
Tol2-mediated large genomic deletions	Transposon element Tol2 integrates into the genome, causing gene disruption	Integration occurs randomly and efficiently, inheritable, huge acceptance in the community, excellent construct availability	Requires large space and is labor-intensive, tends to have hot spots	Wiki and repository: http://www.tol2kit.genetics.utah.edu, constructs available through Addgene
Retrovirus-mediated insertional mutations	Retroviral element integrates into the genome, causing gene disruption	Integration occurs randomly and efficiently, inheritable, rapid identification of affected gene	Same as above	N/A
ENU-mediated targeting induced local lesions in genomes (TILLING)	ENU mutagenesis followed by sequencing genomic region of interest	Generates a diverse range of mutant alleles for genetic analysis	Same as above	http://www.sanger.ac.uk/Projects/D_rerio/zmp/
ZFN-mediated gene targeting	Zinc finger nucleases-mediated DNA double-strand break	Generates small deletions or insertions at desired genomic region of interest	Constructing ZFNs targeting desired target sites is time- and labor-consuming; generation of stable mutants takes up to 12 months	N/A
TALEN-mediated gene targeting	TALEN nucleases-mediated DNA double-strand break	Same as above	Off-target effects; generation of stable mutants takes up to 12 months	N/A

Table 9.2 (continued)

Reverse genetic tools	Mechanism	Pros	Cons	Resources
CRISPR/Cas9-mediated gene targeting	CRISPR/Cas9-mediated DNA double-strand break	Same as above, and the most versatile gene targeting method	Generation of a stable mutant takes up to 12 months; genetic compensation reported [27]	N/A

translational start site to block translation completely or by targeting splice-site junctions in the pre-mRNA causing either exon skipping or intron integration resulting in frameshift and premature stop codon generation [31, 32]. While translation blocking MOs require an antibody specific for the targeted protein to assess efficacy, the efficiency of disruption of splicing by the splice-site MOs can be easily evaluated by RT-PCR. The development of photoactivatable MOs (caged MOs) helped to overcome the limitation that MOs act globally and constitutively [33]. Caged MO activity can be temporarily and even spatially controlled by applying cellular resolution light, i.e., using two-photon microscopes [34]. MOs proved to be a versatile tool not only to inhibit gene function but also to confirm genetic interactions of genes when titrated and co-injected, for gene dosage experiments, to suppress miRNAs by targeting miRNA maturation or for miRNA target validation by interfering with miRNA target 3′-UTR binding [35–37]. Despite being extremely valuable, MOs come with the disadvantage of having transient effects because they do not cause genetic lesions, allowing gene knockdown only for about 3 days [31]. Further, MO experiments require well-trained and experienced staff to control for potential off-target effects and toxicity [30, 38]. Recently, discrepancies between the observed phenotypes induced by MOs and stable mutants were reported, which can be in part explained by genetic compensation mechanisms occurring in mutants but not when the gene is transiently inactivated as in morphants, possibly thereby obscuring phenotypes otherwise visible after MO use [27, 39].

The latest development of advanced genome-editing technologies complements the existing reverse genetics tools in zebrafish. First, zinc finger nucleases (ZFN), then TALE nucleases, and ultimately CRISPR/Cas9 have been shown to be highly effective in zebrafish, and particularly, CRISPR enables cheap and relatively effortless generation of mutants in virtually every lab [40–45]. Narayanan et al. recently demonstrated the feasibility of simultaneous in vivo mutagenesis of entire gene and miRNA gene families by multiplex CRISPR/Cas9, highlighting the versatility of this system [46]. Multiplexed CRISPR/Cas9 was subsequently employed to demonstrate that miRNAs provide phenotypic robustness and protect the vascular systems from changing environmental conditions [47].

Besides inducing simple lesions, CRISPR/Cas9 enables targeted genome editing allowing introduction and correction of distinct mutations, integration of exogenous sequences like LoxP, and even introduction of reporter genes, including GFP [48]. However, besides the very well and efficiently performing NHEJ

(non-homologues end-joining), HDR (homology-directed repair) seems to work rather inefficient in zebrafish [48, 49].

As in other animal models, transgenic strategies are another valuable reverse genetics tool to elucidate gene function. Either wild-type forms or constitutive active or dominant-negative isoforms of the gene of interest can be transiently, by simple RNA injections, or stably, by stably integrated transgenesis, expressed. Generation of a stable transgenic line takes between 6 and 12 months. Here, also a diverse tool set, including GAL4/UAS- and Cre/LoxP-based systems, and a diverse set of promoters, including cell-type-specific promoter elements or tamoxifen and heat shock-responsive elements to drive spatially and temporally controlled expression of the transgene, are available [50].

9.2.3   Genetic Diagnostic Testing Using Zebrafish

Current large-scale genomic sequencing studies of human patients have identified many disease-associated genetic variants at an accelerating pace and at ever-decreasing costs. One of the major challenges that we are facing with all these genomic data is to determine the pathogenicity of newly identified candidate disease genes and genetic variants. This is particularly true for the ones that have not been previously evaluated in an experimental model system. Owing to the abovementioned advantages as a vertebrate model system, zebrafish has emerged as an attractive model to determine the cause-effect relationship between the genetic variants and the human defects and the underlying molecular disease mechanisms.

Giving the versatile genetic tool set in zebrafish, the ability of genetic diagnostic testing ranges from simple gene causality and single variant testing to the identification and characterization of more complex disease genetics. We will focus here on how the zebrafish can be used to test for a variety of genetic testing scenarios; distinct examples can be found in the disease entity sections below.

(I) Using transient MO-mediated knockdown or stable CRISPR/Cas9 knockouts as a tool to identify disease causality of a gene from single genes or from a set of candidates isolated through, i.e., GWAS, enables simple and fast causality screening. Gene candidates mapped for disorders segregating under a recessive mode of inheritance can be easily and rapidly tested. Phenocopy of large parts of the phenotypic spectrum found in the respective patient cohort allows for identification and functional verification of identified null variants with predicted nonsense, frameshift, or splice-site mutations. **(II)** The power of zebrafish is the ability to transiently overexpress genes or gene variants by injection of in vitro generated RNA carrying the desired mutation. This allows very specific in vivo variant testing. In vivo complementation experiments by combining mRNA injection of transcripts carrying the to-be tested mutation with MO-mediated knockdown or stable knockout result in more specific mutation-focused testing. The loss-of-function effect of the variant is proven by the inability to rescue the induced gene loss. This method is very helpful to identify null variants in a scenario of recessively inherited disorder. Injection of variants into wild-type animals enables identification of dominant-

negative acting gene mutations. If small amounts of the mutant variant cause a disease phenotype, while injection of equal amounts of the wild-type variant has no effect, this indicates toxicity of the mutant allele and hence dominant-negative mode of action [51]. **(III)** The ease to generate stable mutants in single or multiple genes at once and the availability of a huge variety of mutant alleles through international resource centers as mentioned above enable even the unraveling of more complex genetic traits in vivo. Combining mutant alleles by multiplex targeting through CRISPR or compound mutation generation by crossing of different mutant lines allows demonstration of genetic interactors and modifiers of phenotypic traits [47, 52]. Convenient is the use of MOs, since here the amount of gene reduction can be titrated. Combining carefully titrated MOs targeting different specific genes can thus be used to prove genetic interaction and disease modifiers in an in vivo setting with little time effort [35].

The applicability of zebrafish in the diverse genetic scenarios is summarized in Table 9.3.

9.3   Cardiovascular Disease Models

9.3.1   Congenital Heart Disease Modeling in Zebrafish

Congenital heart diseases (CHDs) are the leading cause of human birth defects, and many of these diseases originate from genetic defects that impact cardiac development and morphogenesis [58, 59]. During development, the heart is the first organ to form and function. After its initial formation at the ventral midline, the vertebrate heart undergoes a series of complex morphogenetic processes that transform the linear heart tube into a functional pumping organ (Fig. 9.1a–g) [5, 63, 64]. Although the zebrafish heart appears to be structural simpler than its counterpart in higher vertebrates, much of the molecular and cellular mechanisms governing cardiac formation and further maturation are conserved between zebrafish and higher vertebrates [5, 23, 60, 64–66].

CHDs in humans feature a wide variety of structural abnormalities that affect nearly every structure of the developing heart including the myocardium, the valves, and the great vessels [59]. If left uncorrected, in severe cases, the structural defects will lead to cardiac dysfunction and thus negatively impact life quality of the affected individual. In zebrafish, myocardial wall abnormalities manifest as either hypoplastic ventricle or hypotrabeculation, both of which cause progressive reduction in cardiac contractility [67]. Cardiac valve malformations often arise from defective endocardial cell migration and/or remodeling, leading to improper endocardial cushion and/or valve leaflet formation [23, 68, 69]. These defects can result in blood regurgitation and altered intracardiac fluid dynamics [68, 69]. Other cardiovascular defects in zebrafish include heterotaxy whereby the relative position of the zebrafish cardiac chambers is altered [70, 71], and malformations of the pharyngeal arch arteries (PAAs), the transient embryonic blood vessels that eventually give rise to the carotid arteries and great vessels of the heart [72, 73].

Table 9.3 Overview of usage scenarios for genetic diagnostic testing using zebrafish and the available methods

Query	Approach options	Findings
Disease causality testing of a single candidate gene Identification of a disease gene from a set of genes, i.e., by GWAS	*MO (weeks) or stable KO, CRISPR/TALENS (months)* *MO (weeks) or stable KO, CRISPR/TALENS (months)* combined with *transgenic sensor/marker line*	– Identification and validation of a disease gene [51, 53, 54] – Complete phenocopy proves loss-of-function, null variant effect or recessive mode of inheritance [51, 53, 54] – Phenotype in heterozygotes or MO titration proves haploinsufficiency [55] – Isolation of distinct effects and on distinct cell types/tissues/ biological processes [56, 57]
Pathogenicity testing of single identified mutations	WT/variant *RNA injection combined with MO KD or stable KO*	– Inability to rescue with variant mRNA *proves pathogenicity* [57] – Could indicate *recessively inherited* disorder
	WT/variant *RNA injection in WT*	– Development of phenotype with variant injection *proves pathogenicity* [51, 57] – Indicates *dominant-negative* effect of variant [51, 57]
Complex Trait Testing (polygenic evidence) *or* Candidate gene/ signaling network *or* Disease modifier testing	*Multiple mutant/heterozygotes* generation by crossing different mutant lines [52] *Sensitization of a het line* by introduction of a second het mutation [52] *Multiplex CRISPR* targeting [47] *MO combination* experiment [35] *Sensitization* by using *multiple titrated MOs* [35] *Rescue of MO KD* by injecting genetic *interactor or* downstream *affector RNA*	– *Polygenetic cause for a disease* can be proved [52] – Proof for *genetic interaction* [35, 47, 52] – Causation testing of *gene networks* [35, 47, 52] – Identification of *disease modifier* [35, 47, 52] – *Risk factor stratification* [47]

GWAS genome-wide association study, *KD* knockdown, *KO* knockout, *MO* morpholino, *WT* wild type. *Citations provide examples for distinct genetic testing scenarios. The list of cited articles is not exhaustive*

Hypoplastic left heart syndrome (HLHS) is a rare but complex CHD characterized by severe hypoplastic left ventricle and extremely underdeveloped mitral and aortic valves [74, 75]. These severe structural defects render the left side of the heart less efficient in pumping blood to the body, and surgical corrections are needed to improve the survival of HLHS infants [76]. HLHS usually occurs as isolated disease and the associated genetic loci include, among others, those
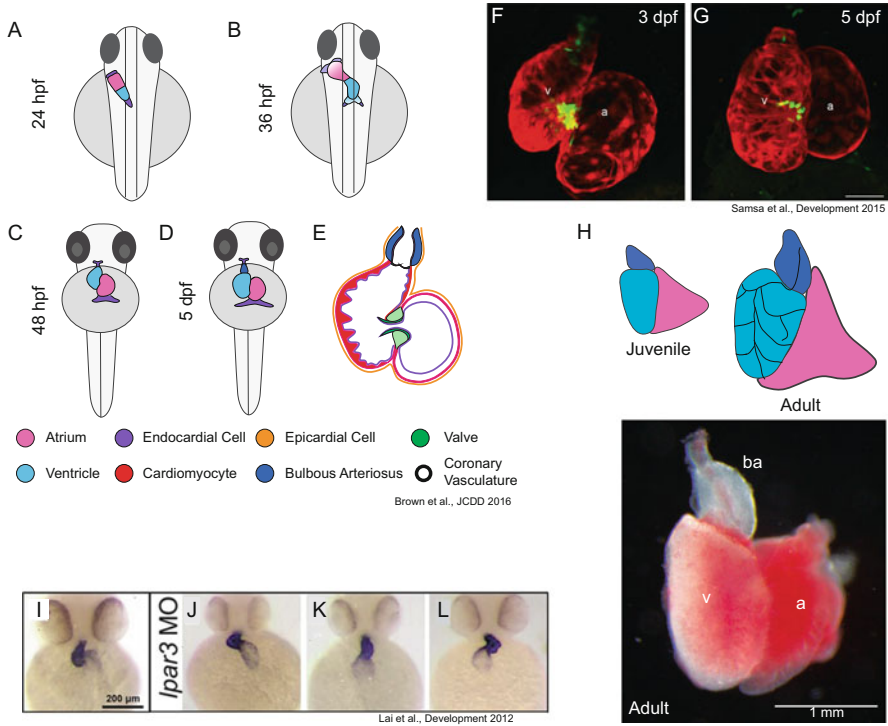
Fig. 9.1 The embryonic and adult zebrafish hearts. (**a**) The zebrafish linear heart tube at 24 hpf. (**b**) The looping zebrafish linear heart tube at 36 hpf. (**c**) The two-chambered zebrafish heart at 48 hpf. (**d**) The zebrafish heart grows in size by 5 dpf. (**e**) Cross-sectional view of 5 dpf zebrafish heart showing ventricular protruding trabeculae, cardiac valves, and the outmost epicardial layer. Modified from Brown et al. JCC 2016 [60]. (**f, g**) Confocal projections of 3 and 5 dpf zebrafish hearts, respectively. From Samsa et al., Development 2015 [61]. (**h**) Schematics of juvenile and adult zebrafish hearts at the top and an isolated adult heart at the bottom. (**i–l**) Control hearts (**i**) and hearts with left-right patterning defects (**j–l**) stained by in situ hybridization labeling the cardiac-specific myosin light chain 2. From Lai et al. Development 2012 [62]

encoding the transcriptional factors NKX2.5 and HAND1 and the gap junction protein GJA1 [77–79]. Interestingly, recent studies in zebrafish revealed an important role for Nkx2.5 and its homologue Nkx2.7 in chamber identity maintenance [80, 81]. Although the numbers of differentiated ventricular and atrial cardiomyocytes (CMs) are not affected by the loss of *nkx2.5* function at the linear heart tube stage, zebrafish *nkx2.5* mutant demonstrates a nearly 50% decrease in the number of ventricular CMs with a corresponding increase in the number of atrial CMs starting from cardiac looping stage. These *nkx2.5* mutant phenotypes are further enhanced by a loss of *nkx2.7* function. These observations suggest that these two Nkx factors play critical role in maintaining ventricular and atrial cell identity during the time of cardiac loop and chamber emergence [80, 81]. Although no studies have reported an enlargement of atria in HLHS human patients, studies
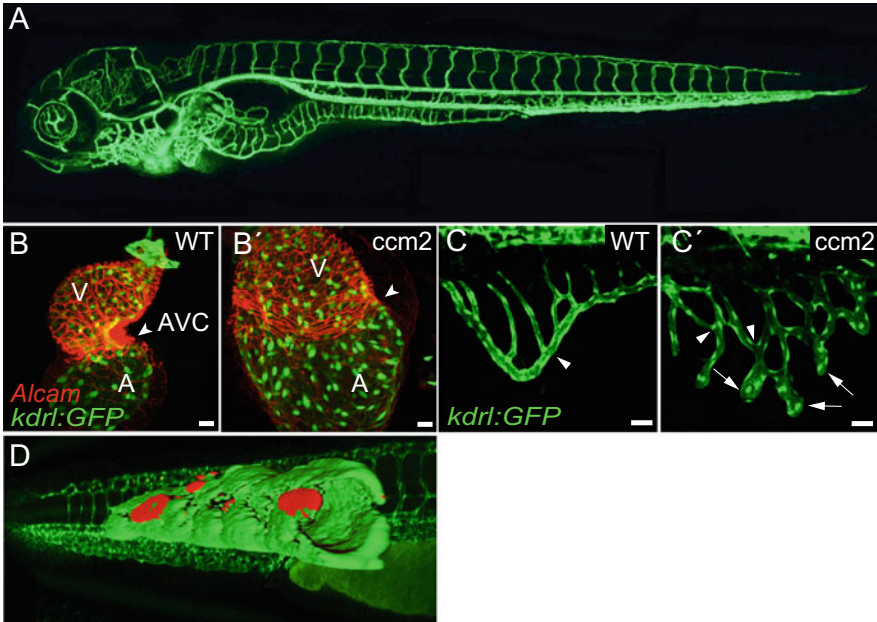
Fig. 9.2 Zebrafish as a vascular model. (**a**) Vasculature of a transgenic Tg(*kdrl:EGFP*) embryo at 5dpf. (**b**, **c**) Cardiovascular defects in zebrafish ccm2 mutants, characterized by endocardial overproliferation (**b′**) and increased sprout- and branch point formation (c′) at 48hpf. From Renz et al. [83] (**d**) Zebrafish as a model for atherosclerosis. Lipid deposits labeled by fluorescent lipids in the vascular wall of Tg(*kdrl:EGFP)* transgenic animal [84]

using zebrafish might still provide mechanistic clue as to why HLHS patients develop miniature left ventricles. Combing mouse forward genetics and further validation by CRISPR/Cas9 gene editing in mouse and zebrafish, a recent study showed that Sap130, a histone deacetylase complex subunit, mediates left ventricular hypoplasia, further demonstrating the value of zebrafish as a powerful genetic system to model HLHS [82].

Cardiac valves are critical components in the heart that function to ensure unidirectional blood flow (Fig. 9.2e). Human patients with congenital valve defects would present either obstructed or backward blood flow due to valvular stenosis and valvular insufficiency, respectively [85–87]. In zebrafish, the atrioventricular (AV) valve that forms at the AV junction is the most well-studied valvular structure [23, 68, 69, 88, 89]. At 48 hours postfertilization (hpf), as the AV junction constricts and demarcates the two cardiac chambers, the endocardial cells at the junction begin to differentiate and form a specialized ringlike structure, the AV canal, to help reduce retrograde blood flow [68, 69]. Around 72 hpf, the AV endocardial cells further invaginate and generate primitive valve leaflets allowing for complete prevention of regurgitation [69]. Due to the conserved molecular mechanisms underlying valve formation between zebrafish and higher vertebrates, zebrafish has been utilized to evaluate the potential in vivo effect of genetic variants associated with human

congenital valve defects. By sequencing 32 candidate genes implicated in atrioventricular septum (AVS) development, a recent study identified 11 detrimental genetic variants associated with AVS defects [57]. Further functional analysis demonstrated that expression of a patient-specific ALK2 L343P variant in zebrafish embryos led to aberrant expression of endocardial cushion marker genes and disrupted AV canal formation. More recently, Durst et al. identified *DCHS1* missense mutations as an inherited risk factor for mitral valve prolapse (MVP) [53]. *DCHS1* encodes a member of the cadherin superfamily involved in cell polarity. MO-mediated knockdown of the zebrafish homologue *dchs1b* compromised AV canal formation and resulted in retrograde blood flow [53]. Intriguingly, the wild-type human *DCHS1*, but not the two *DCHS1 variants* (P197L, R2513H), *rescued the* AV canal defects caused by *dchs1b* knockdown in zebrafish embryos, further supporting the link between the familiar mutations with MVP [53].

Heterotaxy is a rare birth defect featuring randomization of visceral organ situs [90]. Although situs inversus leads to little or no medical problems, heterotaxy often causes structural abnormalities in the heart and/or other major visceral organs. The heart is the first organ that responds to laterality signals and thus exhibits left-right asymmetry (Fig. 9.1h–k). In addition to the positioning defect, other cardiac defects seen in heterotaxy patients are transposition of the great arteries and septal defect [91–93]. Because of an essential role of the motile cilia in left-right patterning, genetic mutations that affect motile cilia formation and/or function and the downstream nodal signaling that controls left-right patterning result in heterotaxy and cardiac structural abnormalities [94]. To date, targeted candidate exome sequencing has identified many human mutations in ciliary dynein components, such as dynein intermediate chain 1 (DNAI1) [95, 96], dynein heavy chain 5 (DANH5) [95], dynein heavy chain 6 (*DNAH6)* [97], and left-right dynein (DNAH11) [96], in heterotaxy patients. Selection of the candidate genes was based on genetic data obtained from model organisms, in particular *Chlamydomonas* mutants with the defective structure and/or function of flagella [98]. Intriguingly, recent human genetic studies have identified novel heterotaxy-causing genes, some of which have unanticipated roles in cilia structure and function or potentially affect the downstream signaling controlling left-right patterning [99].

9.3.2 Cardiomyopathy Modeling in Zebrafish

In 2002, with the zebrafish mutants *silent heart* (*sih*) and *pickwick* (*pik*) harboring mutations in cardiac troponin and titin, respectively, rendering the hearts a contractile and recapitulating human cardiomyopathy, the usefulness of zebrafish as a cardiomyopathy model became obvious [100, 101]. Both genes are associated with human hypertrophic (HCM) and dilated cardiomyopathy (DCM) [102, 103]. In subsequent years, many more cardiomyopathy-causing genes were evaluated using the zebrafish. Roughly 96% of all known human cardiomyopathy-causing genes have a direct zebrafish orthologue, supporting the usefulness of zebrafish [104]. The zebrafish even helped to identify novel DCM genes, i.e.,

Nexilin, which was first identified as a novel component of the sarcomeric z-disk in zebrafish and then confirmed to be a molecular base of human DCM by a genetic screening of patients with idiopathic DCM [51]. Strikingly, even the ultrastructural characteristics of Nexilin loss described in zebrafish were mimicked in human Nexilin mutation carriers. Subsequently, Nexilin was also linked to human HCM [105]. Vogel et al. systematically evaluated the usefulness of zebrafish as a disease animal model for human DCM [106]. They knocked down a diverse set of known human DCM genes and evaluated the resulting phenotypes. Inactivation of all genes not only reliably caused DCM, but zebrafish morphants even recapitulated gene-specific disease characteristics, highlighting the value of zebrafish as a diagnostic model to evaluate human cardiomyopathy genetics. Genes evaluated in zebrafish quickly expanded from merely sarcomeric proteins to signaling molecules, transcription factors, and cytoskeletal and other regulatory proteins [106–111]. Zebrafish models contributed dramatically to novel mechanistic insights of human cardiomyopathy, as exemplified by a study from Zou et al. The authors used CRISPR technology to generate distinct titin mutants, leading to the identification of a human conserved titin internal promoter that can rescue favorably mutations residing in the N-terminus of the protein. This renders N-terminal mutations less severe than mutations affecting the C-terminus and might serve as an explanation for the observed variability in expressivity and severity in human titin mutation carriers [112].

Besides larval zebrafish, adults recently are used to model cardiomyopathy. Human cardiomyopathy usually affects adult individuals with disease mutations predominantly being recessively inherited. While previously homozygous zebrafish mutants with manifestation of disease during larval developmental stages were predominantly analyzed, adult zebrafish might represent a model closer modeling the human pathology. The adult zebrafish heart consists of all cell types found in the human heart, including cardiomyocytes, fibroblasts and endothelial cells, while the larval heart mainly contains cardiomyocytes. Common pathways involved in disease progression in human heart disease, including the β-adrenergic system, were shown to function more similarly in the adult than in the larval heart [113]. A few adult cardiomyopathy models exist that contributed significantly to a deeper understanding of disease progression and potential therapeutic opportunities [114–117]. A recent study aimed at understanding the basis for the heterogeneity of observed phenotypes in essential myosin light chain (MYL3) mutation carriers, which can be observed even in patients carrying the same mutation [118]. The authors used adult, heterozygous mutant fish, mimicking human dominant mutation carriers. Combining functional analyses with molecular and biochemical assays, the study provides evidence that MYL3 is essential to adapt heart function to physical stress. Heterozygous mutant fish developed cardiomyopathy and increased lethality only after exposure to forced swimming while being unremarkable under normal conditions. Variability in expressivity and severity of MYL3-associated cardiomyopathy in humans hence depends on the degree of the stress the heart experiences during lifetime, and controlling cardiovascular stress might represent a possibility to reduce disease symptoms [118].

While being valuable to more closely resemble human cardiomyopathy, using adult zebrafish comes with disadvantages. The biggest disadvantage is time, since generating stable mutants is significantly more time-consuming than MO-based larval studies. Further, analysis of heart function in larvae is very easy and can be performed by using a simple light microscope. Because adult zebrafish lack transparency, more advanced imaging modalities have to be used, demanding higher costs and highly trained personnel. In accordance to mammalian studies, echocardiography was adapted to analyze heart function in adult zebrafish, now allowing the assessment of conventional echocardiographic parameters to advanced analysis of detailed myocardial mechanics deploying modern speckle tracking [118–120]. And last but not least, the ability of using larval zebrafish to perform high-throughput screens (see section below) is unprecedented and is impossible to match with adult zebrafish. Nevertheless, adult zebrafish cardiomyopathy models are rising and will more and more complement larval studies in the future.

9.3.3 Arrhythmias

While murine hearts beat with up to 900 beats per minute (bpm), zebrafish hearts contract with a frequency of 60–100 bpm, thus much more closely resembling heart rates of humans. This resemblance is furthermore evident in the very similar form and kinetic of a zebrafish ventricular action potential and the echocardiogram [11]. Drugs targeting late repolarization channels induce confound arrhythmias in zebrafish while being only marginally functional in mice. Although being very small, the larval zebrafish heart is able to present a variety of different arrhythmic flavors, including tachycardia, bradycardia, atrioventricular block (AV block), atrial fibrillation, and sinus exit block. First evidence for the applicability of zebrafish as an arrhythmia model came from the *island beat* (*isl*) mutant in 2001, harboring a mutation in the cardiac L-type calcium channel [121]. *isl* larvae display a noncontractile ventricle and an asynchronously contracting atrium, resembling atrial fibrillation. *Breakdance* mutant zebrafish (*bre*), carrying loss-of-function mutations in the zebrafish orthologue of the human HERG channel, model long-QT syndrome 2 (LQT2) [122, 123]. *bre* hearts develop a second-degree AV block, with the ventricle skipping every other beat of the atrium (2,1 rhythm). Using a morpholino targeting *kcnh6* or high dosages of HERG-blocking drugs, such as terfenadine or E-4031, enables to induce a 3:1 and 4:1 and even up to a third-degree AV block. *bre* fish are very useful to screen for modifiers of LQT2 in vivo [124, 125]. Besides LQT, with *reggae* (*reg*) mutant zebrafish, one of the first animal models for the human short-QT syndrome 1 (SQT1) was introduced [126]. *reg* mutant fish carry a gain-of-function mutation in the zebrafish HERG channel and display a whole range of phenotypes, including complete cessation of contractility over up to hours, to atrial fibrillation and sinus exit block. A sodium-calcium exchanger (NCX1) deficiency was reported for the *tremblor* (*tre*) mutant that displays chaotic and dyssynchronized cardiac contractions and atrial fibrillation due to abnormal calcium transients [127]. A second *tre* mutant, besides being important for rhythmicity, indicated a

role for NCX1 for normal cardiac development and sarcomere formation, since this mutant displayed malformed hearts and severely disrupted sarcomeres [128].

A direct implication for human genetics came from a zebrafish transgenic line that expressed a mutation in the SCN5A sodium channel frequently associated with conduction disease, sinus node dysfunction, atrial and ventricular arrhythmias, and dilated cardiomyopathy in patients [129]. Mutant SCN5A expressing zebrafish developed bradycardia, conduction system abnormalities, and premature death, suggesting conserved functions in the heart and demonstrating the usefulness of zebrafish as an in vivo screening model to distinguish benign from functional genetic variants found in humans with arrhythmias [129].

9.3.4 Vascular Disease

Zebrafish offers the ability to choose from a diverse range of transgenic reporter lines labeling vascular endothelial cells, lymphatic endothelial cells, or vascular smooth muscle cells and pericytes [68, 130–134]. Combined with its exceptional imaging capabilities, this facilitates unprecedented visualization of organ formation and malformation as well as vascular function and malfunction in vivo (Fig. 9.2a). Studies in zebrafish have contributed greatly to advance our understanding of vascular biology and disease. The prominent role of Notch signaling for arterial-venous specification and its function as a determining factor to select tip and stalk cell fates during angiogenesis was first described in zebrafish [135, 136]. Further, our views of early formation of the first embryonic artery and vein were reinvented with observations in zebrafish that the first embryonic vein forms by selective sprouting of progenitor cells from a common arterial and venous precursor vessel subsequently undergoing fate segregation regulated through the ligand EphrinB2 and its receptor EphB4 [137].

Defects in vascular integrity and resulting hemorrhage were among the first pathologies described in zebrafish, advancing our understanding of factors essential for vessel integrity and of genes involved in human disease conditions involving hemorrhage formation [24, 138–145]. The cerebral cavernous malformation (CCM) protein complex plays a crucial role for normal blood vessel development and vascular integrity [146]. CCMs are vascular lesions characterized by enlarged thin-walled blood vessels and lack of supporting subendothelial cells such as smooth muscle or astrocytic foot processes. In patients, CCMs are primarily found within the neurovasculature of the central nervous system and often cause headaches, seizure, or often lethal cerebral hemorrhages due to a loss of function of at least one of the three genes, KRIT1/CCM1, CCM2/OSM, or CCM3/PDCD10. In zebrafish, *ccm* mutants exhibit proliferation and sprouting defects in endocardial and endothelial cells (Fig. 9.2b, c). Mechanistically, the loss of CCM proteins results in a ß1 integrin-dependent overexpression of the zinc finger transcription factors *Klf2a* and *Klf2b*, which in turn causes an upregulation of endothelial-specific factor *egfl7*, thereby promoting excessive angiogenesis. Pharmacological inhibition of VEGF signaling or *klf2a/b* knockdown by antisense oligonucleotide morpholino injection rescued

ccm mutant cardiovascular defects [83]. Thus, zebrafish can help to further unravel so far unrecognized pathomechanistic insights of human diseases and enables identification of potential therapeutic targets.

Worldwide, atherosclerosis is the leading cause of death. It is a pathological process of inflammation and progressive deposition of cholesterol, cellular debris, and calcium in the artery walls. Zebrafish, with its unique visualization abilities by combining transgenic reporter lines with biological indicator dyes, can help to identify novel mechanisms and candidate genes and their role in the pathogenesis of the disease to ultimately screen for new druggable targets [147, 148]. Recent studies have shown that zebrafish fed with high-cholesterol diet (HCD) mimic lipid deposition within arterial vessels in humans (Fig. 9.2d) [84]. Combined with transgenic lines marking leukocytes or macrophages, zebrafish becomes a powerful tool to analyze inflammatory modulating drugs on the progression of atherosclerosis in vivo [149].

9.4 Zebrafish in High-Throughput Drug Screens

Over the last 100 years or so, classical drug development has been employing two broad types of small molecule screening strategies, the phenotypic screening and the target-based screening, to identify small molecules that can be used as lead compound for novel pharmaceutical drugs [150]. Historically, new drugs discovery has been mostly relied on phenotype-based screening that utilizes cellular or animal models to search for compounds that induce desirable phenotypic change(s). Since 1980s, owing to the advances in molecular biology and genomics, target-based screening, which aims to identify small molecules against defined molecular targets implicated in human diseases, has immediately gained popularity. Nevertheless, there are a few advantages for phenotypic screening over the target-based screening as a tool of choice for lead discovery [151, 152]. For instance, phenotypic screening doesn't require prior knowledge about the molecular target to identify drugs that produce therapeutic effects. This strategy also has the potential to identify compound that alleviates a diseased phenotype through targeting multiple biological targets. As such, the last two decades has witnessed the emergency of zebrafish as an animal model to the forefront of phenotype-based small molecule screening [152].

In 2000, Peterson et al. published the very first whole-organism-based small molecule screen and identified compounds that affected various aspects of early zebrafish development [153]. The same group also performed the first chemical suppressor screen aiming to reverse the coarctation phenotype observed in zebrafish *gridlock* mutants [154–156]. *gridlock* harbors a hypomorphic mutation in the *hey2* gene that encodes a bHLH transcriptional repressor [155]. After screening 5000 small molecules, two structurally related compounds were identified to suppress the *gridlock* coarctation phenotype likely through upregulating VEGF expression [154]. Given the resemblance of zebrafish cardiac electrophysiology to that of human hearts [11], zebrafish has been served as a particularly valuable model for identifying therapeutics that can rescue arrhythmia defects. By screening 1200

commercially available small molecules, Peal et al. identified two compounds that reproducibly rescued *breakdance* mutant long-QT defect [125]. In recent years, phenotype-based screening using zebrafish has benefited significantly from the latest developed motorized robotics systems. These high-throughput tools and technologies facilitate embryo dispensation, compound delivery, and incubation, as well as image acquisition and analysis of a variety of parameters to grasp the complexity of cardiac function, and will undoubtedly accelerate the identification of cardiovascular disease therapeutics in whole-organism-based systems.

9.5   Conclusions

– A variety of human cardiovascular diseases can be modeled in zebrafish.
– Availability of a highly versatile genetic tool set allows easy and fast interrogation of gene function and evaluation of disease causality of gene candidates and even provides insights into functional genetic networks.
– The unprecedented visualization capabilities of zebrafish enable phenotypic characterization from whole organ level down to cell and subcellular characterization.
– As a future prediction, zebrafish will urge into high-throughput drug discovery and will accelerate the identification of disease, including cardiovascular disease, therapeutics in whole-organism-based systems in the future.

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