

LITTLE FISH, BIG DATA: ZEBRAFISH AS A MODEL FOR CARDIOVASCULAR AND METABOLIC DISEASE

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Gut P, Reischauer S, Stainier DYR, Arnaout R. Little Fish, Big Data: Zebrafish as a Model for Cardiovascular and Metabolic Disease. *Physiol Rev* 97: 889–938, 2017.

Published May 3, 2017; doi:10.1152/physrev.00038.2016.—The burden of cardiovascular and metabolic diseases worldwide is staggering. The emergence of systems approaches in biology promises new therapies, faster and cheaper diagnostics, and

personalized medicine. However, a profound understanding of pathogenic mechanisms at the cellular and molecular levels remains a fundamental requirement for discovery and therapeutics. Animal models of human disease are cornerstones of drug discovery as they allow identification of novel pharmacological targets by linking gene function with pathogenesis. The zebrafish model has been used for decades to study development and pathophysiology. More than ever, the specific strengths of the zebrafish model make it a prime partner in an age of discovery transformed by big-data approaches to genomics and disease. Zebrafish share a largely conserved physiology and anatomy with mammals. They allow a wide range of genetic manipulations, including the latest genome engineering approaches. They can be bred and studied with remarkable speed, enabling a range of large-scale phenotypic screens. Finally, zebrafish demonstrate an impressive regenerative capacity scientists hope to unlock in humans. Here, we provide a comprehensive guide on applications of zebrafish to investigate cardiovascular and metabolic diseases. We delineate advantages and limitations of zebrafish models of human disease and summarize their most significant contributions to understanding disease progression to date.

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I. INTRODUCTION

“What is truly revolutionary about molecular biology in the post Watson-Crick era is that it has become digital.”

—Evolutionary biologist Richard Dawkins, 1941

Over the past century, storage of electronic data as binary code and discovery of the genetic code have revolutionized biology. Biology can now be quantified: over 700 animal genomes sequenced, ~20 thousand genes in a human genome and ~1.25 gigabytes to store it; over a quarter of a million personal genomes sequenced (282). Just a few decades ago, biologic studies were largely characterized by relatively small sample sizes and single-gene approaches painstakingly studied in animal models, with broader studies taking advantage of cell culture, *in vitro*, or *in silico* data. Animal models remain indispensable for elucidating mechanism and pathophysiology, with different models best suited for different biological questions. Today, at the intersection of bench science and digital biology, the zebrafish

animal model is poised to be ever more important as a high-throughput vertebrate model suitable for many questions in fundamental research, toxicology, or translational medicine.

Indeed, the zebrafish model system rose to prominence due to its utility in systematic, large-scale approaches to dissect genetic pathways during development (86, 109, 129). In the early 1990s, after large-scale screens had been established in *Drosophila* for a decade, the same researchers who revolutionized our understanding of the genetic control of the body plan of the fly learned of the work of George Streisinger, who pioneered the use of zebrafish, or *Danio rerio*, as a laboratory animal (120, 379). Streisinger showed that adult zebrafish, measuring just 2–3 cm in length, could be housed quite efficiently: up to 30 individuals in a tank the size of a standard mouse cage. He noted that zebrafish’s fecundity, external fertilization, and optical transparency of their embryos made them ideal for studying early development. Laboratories in Tübingen and Boston soon used zebrafish to carry out the first ever large-scale forward genetic screens in a vertebrate, identifying genes critically involved in the development of several organ systems (34, 86, 129, 130, 136, 176, 270, 292, 312, 338, 365, 370, 418).

During this pioneering effort, the roles of over 1,500 mutations in 400 genes were characterized mainly by visually examining developmental defects, taking advantage of the zebrafish's rapid development, optical clarity, and anatomical resemblance to higher vertebrates. Soon, it became evident that mutations could be found in zebrafish orthologs of genes disrupted in human diseases, and, most importantly, that phenotypic characteristics of these mutant zebrafish resembled clinical hallmarks (48, 85, 419). Therefore, although studying the genetics of embryonic development and organogenesis have remained the principal research use of zebrafish, the same traits that originally led to its selection as a model are also making zebrafish an

increasingly important animal model for studying human physiology and disease (19, 81, 212).

Advancements in genome engineering technologies and the emergence of zebrafish core facilities at many universities around the world make zebrafish a widely accessible model with easy-to-use genetics, lower cost, and fewer ethical restrictions than for mammalian models. Zebrafish research is increasing both as a percentage of publications in MEDLINE and as a percentage of National Institutes of Health (NIH)-funded R01 grants (199) [FIGURE 1]. In addition to universities, many pharmaceutical companies have established zebrafish research to assist in drug discovery, includ-

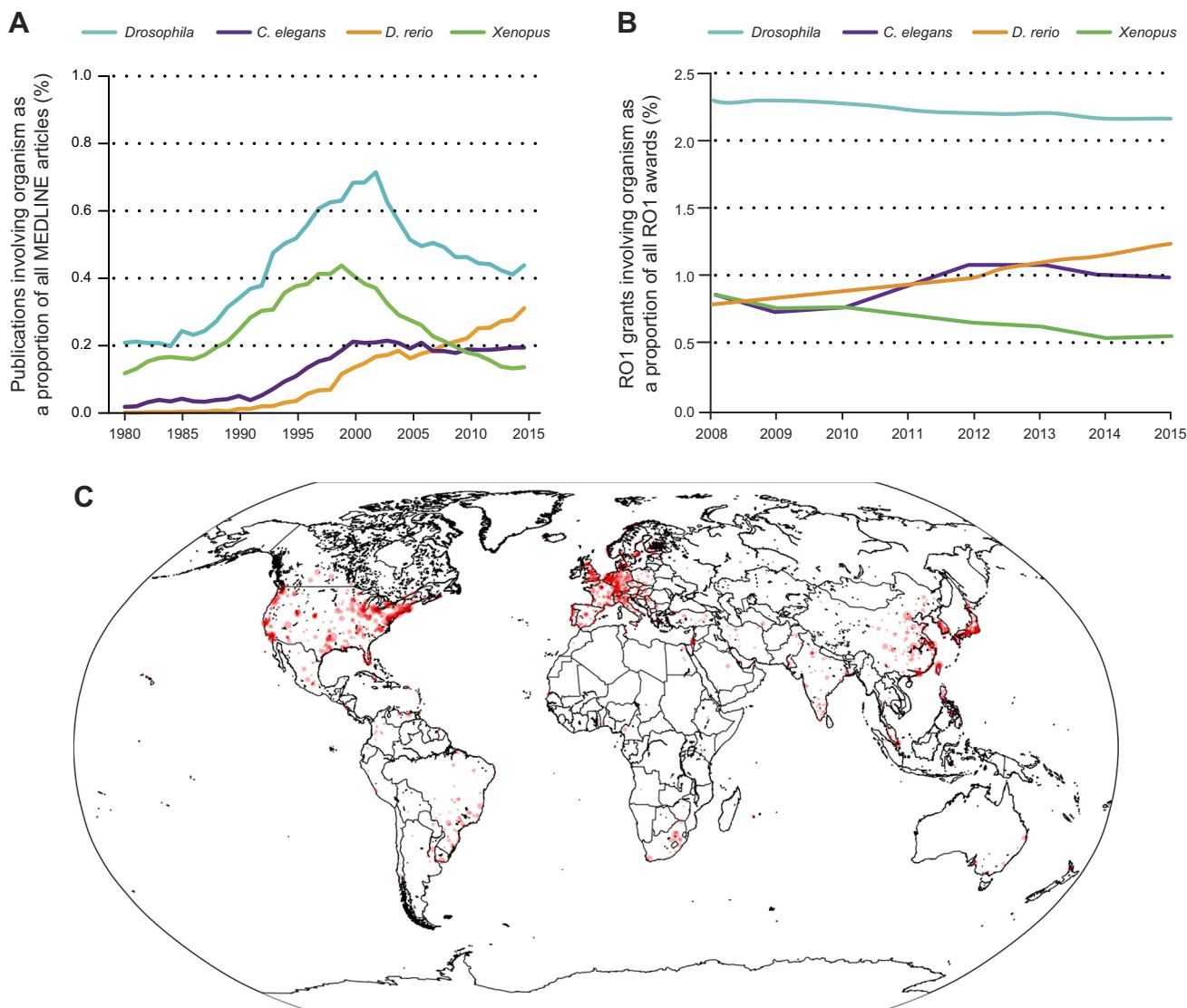


FIGURE 1. Size and shape of zebrafish research. *A*: percentage of publications involving the four most frequently used "lower" organisms *Drosophila*, *C. elegans*, *Danio rerio*, and *Xenopus* as a proportion of all articles published in MEDLINE. After the publication of the initial large-scale forward genetic screens in 1996, the zebrafish became increasingly popular as a model organism. *B*: percentage of NIH R01s involving zebrafish research is on the rise. [Graph modified from Lauer (199). Reprinted by permission from Macmillan Publishers Ltd. Data from *Nature/The week in science*: 5–11, August 2016; reproduced with permission from the NIH Office of Extramural Research/Open Mike blog.] *C*: geotagging authors' affiliations from zebrafish papers in a MEDLINE search shows where in the world zebrafish research is taking place.

ing small molecule and target discovery, toxicology studies, as well as preclinical testing (226, 443). Several small molecules initially discovered in zebrafish screens have entered clinical trials, and more are expected to follow (67, 211, 226, 442). Here, we review state-of-the art approaches in zebrafish research as well as its translatability for human disease with a special emphasis on cardiovascular and metabolic disorders. As part of this introduction, we summarize the key features of zebrafish as an animal model, and we draw a laboratory perspective delineating the advantages and limitations of working with zebrafish.

A. Early Development

Strengths of zebrafish for research in development, drug discovery, and disease must be viewed within the context of other useful models and of the zebrafish's place in the phylogenetic tree. Zebrafish are part of the minnow family of teleost fish, an infraclass of ray-finned fishes that is thought to have arisen ~340 million years ago (156). Basic organ patterning is conserved among vertebrates, and therefore, zebrafish are useful for study of brain, eye, blood, gut, vessels, endocrine, and heart among other organ systems. Unlike other vertebrate models, zebrafish embryos develop a complete body plan with major organ systems, including a beating heart, major vessels with circulating blood, and a rudimentary gut, within 48 hours post fertilization (hpf) (182). Importantly, in addition to this rapid anatomical development, neuronal, hormonal, and paracrine feedback loops are established and become critical for homeostasis at early stages during development (171, 283, 342).

While cardiac and metabolic organ systems form early, the embryo initially relies on passive diffusion from water for oxygen, and on their yolks for nutrients; therefore, even experimental manipulations that severely perturb cardiometabolic organ systems can be studied (370). Furthermore, their short 2- to 3-mo generation time makes zebrafish faster to work with than most other vertebrates.

In addition to developing rapidly, zebrafish also develop externally, a great advantage over mammalian systems for imaging during development. Indeed, a myriad of reporter lines exist to image not only zebrafish morphology but also physiology such as electrical conduction, myocardial contraction, and more (9, 244, 317). Translucent embryos and larvae are amenable to fluorescent, confocal, time-lapse, and also three-dimensional tomographic imaging (160, 244, 317). External development also facilitates transplantation experiments, an often laborious but very powerful technique to investigate questions of cell autonomy, a technique that has been expanded recently to transparent adult zebrafish (54, 218, 432).

B. Scale and Size

Zebrafish are small, but many, making them suitable for a range of assays. Manipulation of the zebrafish genome is fairly straightforward, as will be covered later. One pair of zebrafish can lay ~200 eggs in a single mating. Therefore, hundreds or even thousands of embryos from several mating pairs can quickly be collected and analyzed in a single experiment (**FIGURE 2**). This scale of production lies in contrast to the single-digit litters of murine models, for example, and allows investigation of rare genetic events and parallel hypothesis testing. The scale of zebrafish embryo production and ease of imaging have spawned technologies for automated embryo and larval phenotyping via high-throughput fluorescence biosorting and other approaches (10, 293, 309, 412).

The small size zebrafish embryos and adults can also be an advantage in the laboratory setting. Drug screens and other experiments can be set up without using a large amount of potentially precious reagents. Small size also facilitates whole-tissue (41), whole-organ, and whole-organism transcriptomic (170, 204, 318), proteomic (274), and other 'omics analyses (105), as well as whole organ clonal analysis (102, 122), cell-cell connectivity mapping (353, 366), and mosaicism analysis (54). Instead of analyzing only a biopsy of a larger heart for a given experimental condition, for example, an entire zebrafish heart chamber—or even the entire zebrafish heart—can be submitted for expression profiling. Combining whole-organism as well as whole-organ RNA expression with imaging, Junker et al. (170) created a RNA expression map of the entire zebrafish embryo by performing RNA-Seq on cryosectioned embryo samples along three dimensions. In another elegant study, McKenna et al. (240) recently used CRISPR genome editing to introduce progressive, combinatorial mutations in the zebrafish genome to create DNA "barcodes" used to perform organism-wide lineage tracing, sampling ~200,000 individual cells to reconstruct a fate map.

C. Limitations

Of course, some of the same potential strengths of the zebrafish model can also be drawbacks. Certain organ systems, such as the respiratory and reproductive ones, differ morphologically from those of humans. The small size of embryos and larvae that can be a benefit for some experiments can make it difficult to collect adequate amounts of tissue for others. Although growing, the number of zebrafish-specific antibodies for immunohistochemistry and the utility of zebrafish cell culture are limited compared with those for other animal models. While facilitating experiments involving water-soluble drug administration or biosorting, for example, the fish's aquatic habitat can complicate certain other assays, such as EKG measurements. Finally, while zebrafish breeding is faster than for some other models, the timeline for generating mutants and

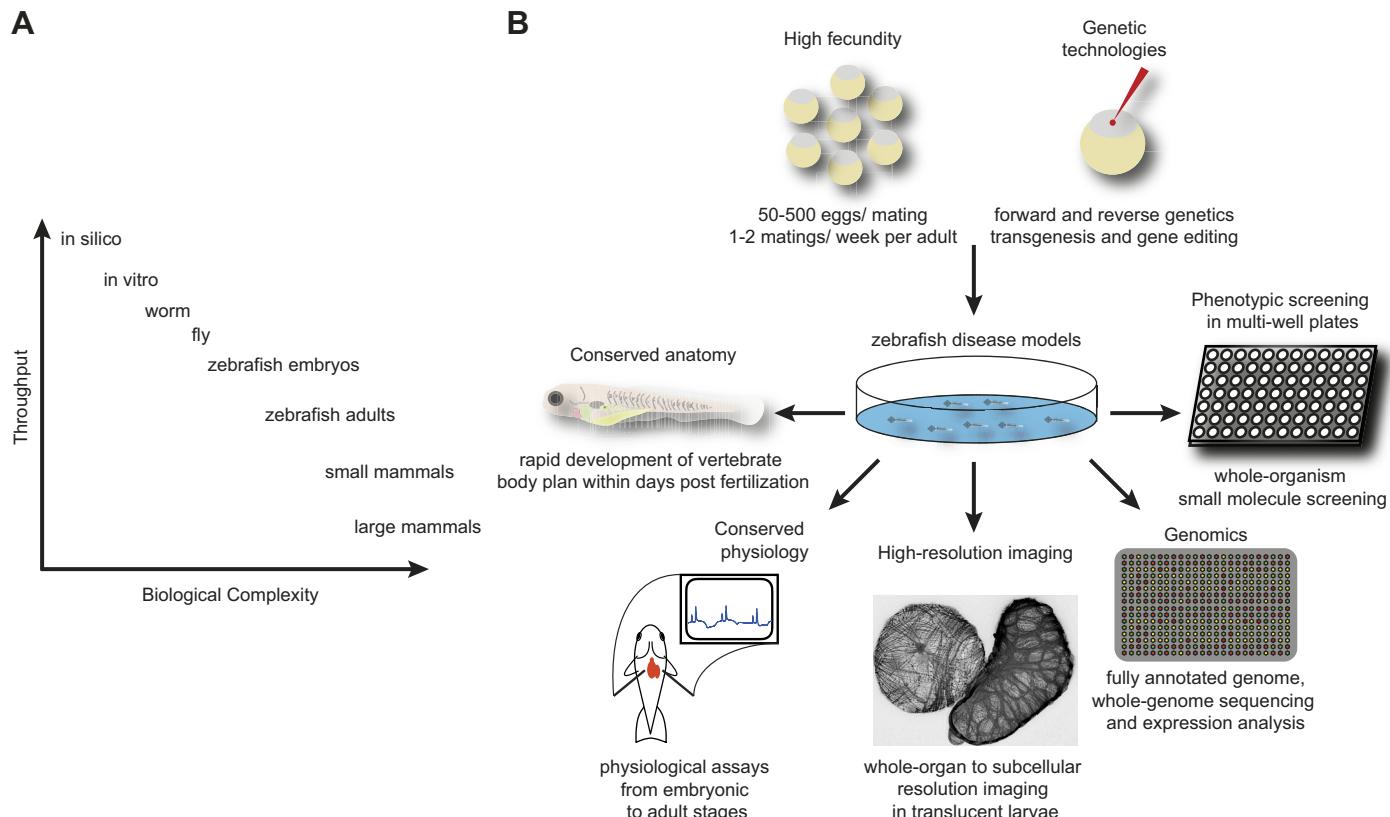


FIGURE 2. Advantages of zebrafish for biomedical research. *A*: zebrafish occupy a unique position in biomedical research as a vertebrate organism accessible to large-scale genetic and chemical screening. *B*: zebrafish are amenable to a broad spectrum of genomic, physiological, imaging, and small-molecule screening approaches.

transgenics is a critical consideration. To generate a stable transgenic adult or homozygous mutant embryos, ~4–6 mo are needed, although several strategies exist to accelerate this process for preliminary phenotyping (39, 404). Careful consideration of genetic strategies for creating an ideal model, and use of the many existing zebrafish resources and repositories (TABLE 1), can facilitate this process, as will be discussed in further detail in section II. Bearing in mind that every model system has some disadvantages, the numerous strengths of the zebrafish model from the laboratory perspective discussed above lay the groundwork for the design of disease and drug discovery models for which zebrafish is best suited.

II. THE GENETIC TOOLBOX FOR ZEBRAFISH RESEARCH

“What I cannot create, I do not understand.”

—Physicist Richard Feynman, 1918–1988

Biomedical researcher and entrepreneur Craig Venter coded this message into the DNA of a synthetic bacterium in a statement on the power of genome engineering (116). Biologists have developed a wide array of strategies for perturbing, and engineering, the genome; these tools are now a foundation to generate zebrafish disease models.

Easy breeding and injection of nucleic acids into the large, externally fertilized zygote make genetics in zebrafish relatively straightforward (FIGURE 2). A vast array of mutants, morphants, and transgenic animals in the literature attest to zebrafish’s genetic tractability (12, 22, 267, 349, 352, 392). To understand genomic engineering in zebrafish, it is important to understand the landscape of the zebrafish genome. First, zebrafish have a diploid genome. Second, zebrafish do not usually thrive when they are inbred, though isogenic lines have been generated. Third, teleosts underwent a whole-genome duplication event ~270 million years ago (150, 161). As a result, ~47% of human genes have a single zebrafish ortholog, while ~24% of human genes have more than one zebrafish ortholog (156). The remaining human genes have no recognized zebrafish ortholog to date, although improving sequence homology algorithms or focusing on functional homology over sequence homology should lead to recognition of more orthologs (TABLE 2).

In some cases, multiple zebrafish orthologs confer a redundancy in gene function that can confound knockout models of human disease; a reported example is the redundant roles of zebrafish *gata5* and *gata6* in cardiomyocyte specification (151). In other cases, having multiple zebrafish orthologs for a single human gene has proven advantageous as they

Table I. Useful resources for zebrafish research

Resource	Description	Website
Ensembl	Genome database for vertebrates and other eukaryotic species	www.ensembl.org
UCSC Genome Browser	Reference sequences and working draft assemblies of a large collection of genomes	http://Genome.ucsc.edu
NCBI Genome Browser	Genome database	https://www.ncbi.nlm.nih.gov/genome/
UniProt	Database for protein sequences and functional domains	www.uniprot.org
Zfin Zebrafish Model Organism Database	In situ hybridization atlas, genetic tools and genomics databases, developmental stages, publications; community website	http://zfin.org
Zebrafish International Resource Center	Stock center for zebrafish lines and plasmids in North America; fish pathology and health services	www.zirc.org
China Zebrafish Resource Center	Stock center for zebrafish lines in China	http://En.zfish.cn
Japanese Zebrafish Resource Center	Stock center for zebrafish lines in Japan	shigen.nig.ac.jp/zebra/index_en.html
European Zebrafish Resource Center	Stock center for zebrafish lines and plasmids in Europe; sequencing, genetic and chemical screening services	www.ezrc.kit.edu
Zebrafish Gene Trap and Enhancer Trap Database	Repository of gene trap and enhancer trap lines, including representative images of each line	Kawakami.laboratory.nig.ac.jp/ztrap/
zfishbook International Zebrafish Protein Trap Consortium	Repository of expression-tagged, revertible mutations in zebrafish genes using gene-breaking transposons	zfishbook.org/
Digital Fish	Repository of flip trap zebrafish lines	Fliptrap.ucsc.edu/static/fliptraptech.html
CreZoo	Database for zebrafish Cre driver lines	crezoo.crt-dresden.de/crezoo/
The Zebrafish Brain Browser	3D Anatomy tool to visualize transgene and gene expression patterns in zebrafish larval brains	science.nichd.nih.gov/confluence/display/burgess/Brain+Browser
ZInC	Zebrafish insertional mutant collection. Joint efforts of UCLA and NIH to generate a genome-wide knockout resource for zebrafish	https://research.nhgri.nih.gov/ZInC/
Zebrafish Neurophenome Project	Resource on neurobehavior and physiological data of adult zebrafish models	kaluefflab.com/znpindex.html
Bio-Atlas Zebrafish Anatomy Atlas	Hematoxylin & eosin histological slides of zebrafish from embryonic to adult stages at anatomical resolution	Bio-atlas.psu.edu/zf/
Sanger Zebrafish Genome Project	Zebrafish genome sequencing project	www.sanger.ac.uk/science/data/zebrafish-genome-project
DANIOCODE	Annotation of functional elements across the zebrafish genome	www.birmingham.ac.uk/generic/danio-code/index.aspx
CRISPR Design	Crispr design tool; scoring tool and calculation of off-target probabilities	crispr.mit.edu
CRISPRz	Database of validated CRISPR targets in zebrafish; database for methods and protocols related to the generation of zebrafish mutants	https://research.nhgri.nih.gov/CRISPRz/

Continued

Table 1.—Continued

Resource	Description	Website
crisprScan	CRISPR design tool; scoring tool and calculation of off-target probabilities	www.crisprscan.org
BROAD Institute Genetic Perturbation Platform	Crispr design tool; gRNA efficiency scoring tool	www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design
TALEN Design	TALEN and CRISPR design tool, including design of CRISPR Cas9 Nickase strategies	http://talendesign.org
Addgene	Repository for plasmids; distribution to academic laboratories only	www.addgene.org
BACPAC Resource Center	Repository for bacterial artificial chromosomes; not-for-profit distribution	http://Bacpac.chori.org
JOVE	Video-based protocols with a large number of protocols related to standard and specialized zebrafish procedures	www.jove.com

have evolved to have different tissue specificities. For example, knockout of the mouse sodium-calcium exchanger gene *Ncx1* leads to embryonic lethality (49); in contrast, zebrafish have two *ncx1* orthologs, one of which is heart specific, and so an *ncx1* mutant was identified in a forward genetic screen for cardiac defects. Zebrafish was therefore a useful model to study the cardiac role of *ncx1*, showing its effect on cardiac calcium homeostasis, heart rhythm, and contraction (88, 197). Also, a whole-organism approach is necessary when the cause of cardiomyopathy is extracardiac, such as with high-output heart failure caused by anemia (114, 295, 407). The genetic tools most commonly used to manipulate the zebrafish genome are described in this section (**FIGURE 3**).

A. Forward Genetics

Insights from forward genetic screens were one of the first demonstrations of the advantages of applying large-scale gene interrogation techniques to biological problems. One of the first researchers to use forward genetics was Thomas Hunt Morgan (1866–1945), who followed the segregation of spontaneous phenotypes in a population of inbred fruit flies and organized them on a genetic map (256). In the late 1970s and early 1980s, Christiane Nüsslein-Volhard and Eric Wieschaus revolutionized forward genetics in fruit flies by using chemical mutagens that induce random mutations within an organism to identify most genes involved in a specific developmental process, a method known as saturation screening (281). Saturation screens are based on two simple ingredients: an animal with a diploid genome which can be mutagenized in high numbers by mutagenic chemicals like *N*-ethyl-*N*-nitrosourea (ENU) or insertional mutagenesis with transposable elements or retroviruses, com-

bined with a simple phenotypic readout. This powerful combination led to the discovery of virtually every gene regulating body plan segmentation in the fly, earning Nüsslein-Volhard and Wieschaus a Nobel Prize in 1995 (4, 280). Many successful screens were also carried out in models like yeast and worms (139, 278). Vertebrate forward genetics remained elusive, however, due to unsuitable breeding schemes (chicken), unsuitable genomic architecture (the allotetraploid *Xenopus laevis*), unsuitability for large-scale husbandry (mouse, chicken), or difficulty in scoring early developmental phenotypes (mouse).

1. Genetic screens in zebrafish

This situation changed fundamentally with the establishment of the zebrafish as a genetic model organism (379). Zebrafish embryos fulfill all genetic, logistical, and phenotypic criteria essential for a successful genetic screen, as mentioned above. Consequently, a wealth of zebrafish screens have identified mutants with highly interesting phenotypes (2, 44, 86, 297, 370). Intriguingly, even screens scoring fish-specific phenotypes have led to the discovery of human disease-associated genes, as fish and human organ systems rely on the function of similar cell types (271). Protocols for efficient zebrafish mutagenesis have been optimized over the years (324), reaching yields of one to two loss-of-function alleles per 1,000 screened genomes for average-sized genes, and show the feasibility of saturation screens if the phenotypic readout is chosen carefully. In other words, to find a potential loss-of-function allele, an average of 1,000 F1 germlines have to be screened. While the first genetic screens in zebrafish relied on relatively simple readouts like body axis formation, skin integrity or the establishment of circulation, the availability of transgenic lines which can serve as markers of specific cell types like

Table 2. Comparing technologies in zebrafish, mouse, and human

Feature	Zebrafish	Mouse	Human
Genome size*	1.4×10^9 bp	2.7×10^9 bp	3.0×10^9 bp
Genome assembly	High quality (GRCz10)	Very high quality (GRChm38)	Very high quality (GRCh38)
Number of genes*	41,154; 26,373 protein coding	46,062; 22,493 protein coding	54,220; 20,433 protein coding
Genes shared with humans	73%	88%	100%
Forward genetics	Established	Limited by cost and logistics	N/A
Reverse genetics	Established; TALEN, CRISPR/Cas9	Established; homologous recombination, TALEN, CRISPR/Cas9	N/A
Knockdown technologies	Established; easy-to-use, cost-efficient with morpholinos	Established; mostly viral delivery of RNAi constructs with variable efficiency depending on the tissue	Viral and nonviral methods feasible for some tissues as part of gene therapy strategies
Conditional alleles	Limited; difficult to insert Lox sites into genetic loci	Established; available for many cell types	N/A
Genome engineering	Established	Established	Proof-of-concept for correction of mutations in hematopoietic cells for human blood disorders; established for mutations in iPSC cells for in vitro studies
Cell culture	Limited: few cell lines available; possibility to culture different cell types	Cell lines; induced pluripotent stem cells; embryonic stem cells; primary cells well established	Cell lines; induced pluripotent stem cells; embryonic stem cells; primary cells well established
Antibodies	Limited	Broadly available	Broadly available
Imaging	Established; high-resolution <i>in vivo</i> imaging with standard confocal microscopy	Limited; <i>in vivo</i> imaging feasible at some stages and in some tissues, but requires highly sophisticated set-ups	Limited to clinical imaging and pathology
Physiology	Limited due to small organ size	Established	Established
Phenotypic small molecule screening	Established; large-scale screening <i>in vivo</i>	Limited to <i>in vitro</i> screening	Limited to <i>in vitro</i> screening

*Numbers were obtained from annotation releases z105, m106, and h108 of the NCBI Eukaryotic Genome Annotation Pipeline: https://www.ncbi.nlm.nih.gov/genome/annotation_euk/. The total number of genes includes coding, noncoding, and pseudogenes.

microglia, pancreatic cells, blood or liver cells have greatly enhanced the resolution of structures accessible to forward genetic screens (297).

Phenotype-based forward genetic screens have specific advantages and disadvantages over reverse genetic approaches. A clear advantage is that any identified mutant in a phenotypic screen is by definition relevant to the developmental process under study. While there may be some bias in the genomic location of mutations especially in the case of insertional mutagenesis, forward genetic screens are by definition random and thus independent of hypothesis-driven decisions that can be based on wrong assumptions. Furthermore, forward genetic screens allow the identification of a range of alleles including hypomorphs, when null alleles can be lethal before the desired biological process can be studied (80). A challenge of forward genetic screens has been the need for efficient protocols to isolate the mutations causing the phenotypes. While in most cases these procedures have been relatively straightforward and as of late have become much more efficient, depending on the chro-

mosomal location, cloning can pose a significant bottleneck (318, 372).

2. Positional cloning

Traditionally, cloning was performed using a large set of known simple sequence length polymorphism (SSLP) markers, or z markers, which were identified and mapped to the zebrafish genome. This work was further extended by the use of radiation hybrid mapping (112), leading to a countless number of precisely mapped SSLP markers. Briefly, mutant zebrafish are sorted by phenotype and subsequently genotyped with various SSLPs. Linkage of an SSLP to the mutation indicates physical proximity. Based on these results, a genetic map is constructed resulting in an interval that is progressively narrowed down to the location of the causal mutation. After genotyping between 1,500 and 2,000 animals and fine mapping, candidate genes within the small remaining interval can be sequenced to look for mutations. Fortunately, the z marker collection is extensive and can be accessed via ZFIN, the zebrafish model organism

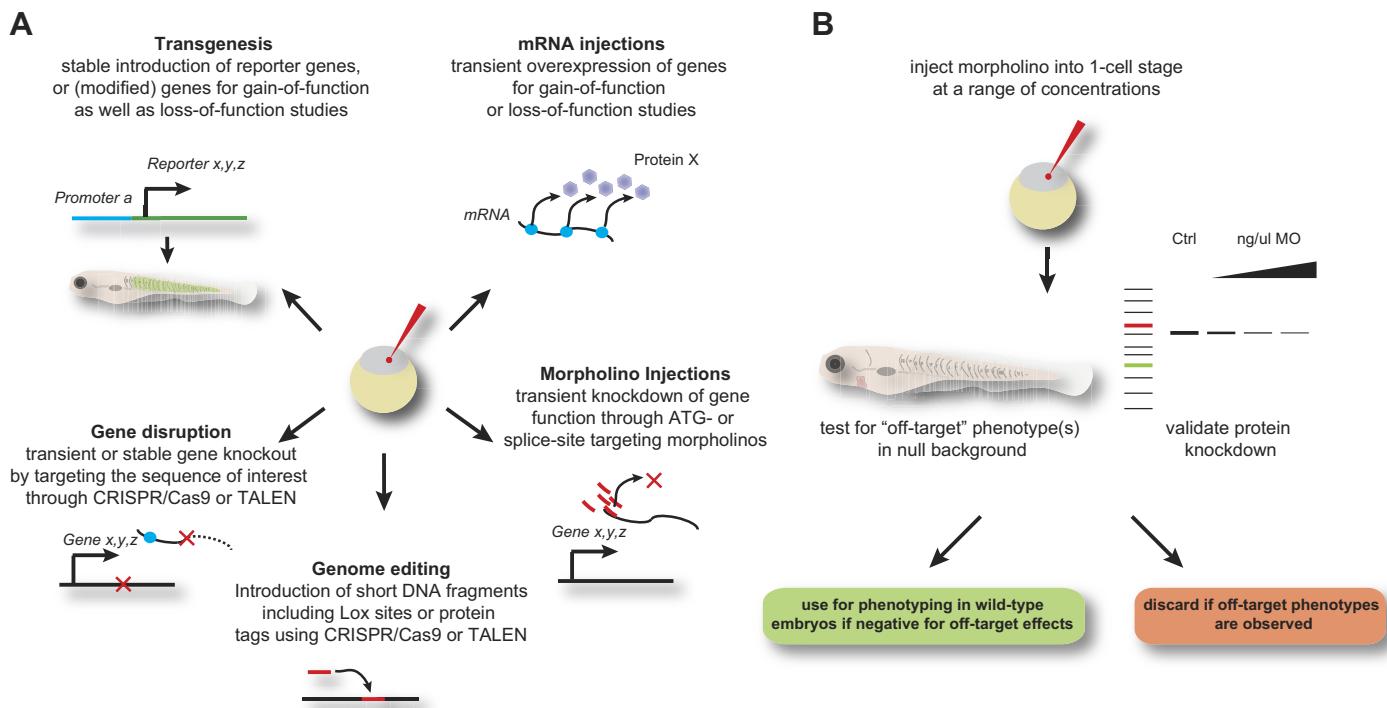


FIGURE 3. Genome manipulations. *A*: overview of technologies to manipulate the zebrafish genome. *B*: guidelines for the correct use of morpholino (MO)-mediated gene knockdown technology. The current recommendation to identify a MO with minimal off-target effects is to test it in a null mutant background, as it should not induce any additional phenotypes.

database. Many SSLP markers have also been mapped to the current assembly of the zebrafish genome (GRCz10) available at <http://www.ensembl.org/>. ZFIN, ENSEMBL, and other important databases and resources for zebrafish research are summarized in **TABLE 1**.

3. Advances in screening and cloning

Incorporating newer genomic techniques into forward genetic approaches has expanded their utility. For example, in contrast to chemical- or radiation-induced mutagenesis, insertional mutagenesis can generate loss-of-function alleles by insertion of a transgene that disrupts gene function (transgenesis will be discussed in detail below). The disrupted gene can usually be easily recovered by sequencing around the inserted DNA fragment, and insertional mutagenesis has been successfully established through the use of retroviral vectors (2, 109, 215) as well as the use of transposable elements (362). Insertional mutagenesis has been further developed with the use of a reporter transgene, which has facilitated the analysis not just of coding but also of noncoding regions such as enhancer elements (55, 76, 290, 310, 395). Insertional mutagenesis offers the significant advantage of rather straightforward identification of the insertion site, but insertional bias limits the number of targetable genes. Several protocols to identify the disrupted genes including inverse PCR and adapter ligation-based PCR have been established for zebrafish and other organisms (399).

While traditional mapping using polymorphic markers remains an important tool, next-generation sequencing technology now significantly facilitates the isolation of the phenotype causing mutations (32, 207, 249, 329). Mapping and identification of mutations affecting particular physiological or morphological processes using whole-genome sequencing (WGS) relies on the same basic principles as traditional protocols, namely, linkage of SNPs to a phenotype. The considerable size of the zebrafish genome in combination with a lack of isogenic lines initially delayed the establishment of WGS as a reliable tool to map mutations cost effectively. Progress in sequencing technology, multiplexing, and cost reduction eventually made WGS an efficient method to map mutations, which is now widely used in zebrafish research. Recent studies show that even multiplexing and low coverage WGS of DNA pools of phenotyped mutants allows direct identification or precise mapping of previously unknown causative mutations (32, 140, 207).

B. Tools for Reverse Genetics

1. Morpholino antisense technology

Zebrafish first gained prominence as a vertebrate model because of the successful forward genetic screens, but in recent years they have become an attractive model for reverse genetics as well (**FIGURE 3**). The global or tissue-specific expression of proteins with dominant negative func-

tion was the principal method of functional knockdown in the early 90s and remains an excellent tool for functional studies. By the late-1990s, however, antisense oligonucleotides joined the toolbox. These small, synthetic single-stranded DNA fragments hybridize to specific regions of an endogenous mRNA and are easy to use in zebrafish because they can be injected into embryos in a high-throughput fashion and at variable concentrations (**FIGURE 3**). The development of morpholino-modified oligonucleotides (simply called “morpholinos”) dramatically improved their stability against nucleases and degradation, so the use of morpholinos became the standard approach for gene knockdown in zebrafish (267).

Morpholinos work by hybridizing to a defined target sequence. However, they become diluted as cell divisions in the growing zebrafish embryo continue, mostly limiting their effectiveness to the first 2–3 days of development. Morpholinos are most effective when targeting the translational start site, thereby inhibiting protein synthesis. Other morpholinos are designed to target a splice acceptor or donor site, leading to exon skipping or intron retention which in turn can cause frameshifts, insertions, missense, or nonsense mutations within the target gene coding sequence. Translation-inhibiting and splice-targeting morpholinos each have their own advantages and disadvantages. The use of splice site morpholinos are by definition limited to targets that contain introns, and they have no effect on mature mRNAs (from maternal contribution for example). Therefore, morpholinos targeting the translational start site usually work more efficiently. On the other hand, evaluation of the effectiveness of translation-inhibiting morpholinos is significantly more difficult. The only valid test for translational inhibitors is to quantify the gene product, which is often problematic due to the lack of specific antibodies against zebrafish proteins. For splice-targeting morpholinos, quantification of mature versus mis-spliced mRNA by RT-PCR is easily done (**FIGURE 3**).

The specificity of morpholinos is a concern recently revived following direct comparison of phenotypes in morpholino-injected embryos/larvae versus genome-edited mutants generated via transcription activator-like effector nucleases (TALENs) or clustered regularly interspaced short palindromic repeats (CRISPRs) (188, 200, 277, 325). In most cases reported so far, there is a clear discrepancy between these phenotypes with the mutants exhibiting less severe phenotypes, leading to vigorous debate within the fish community, as well as others, as to whether morpholino knockdown of gene function should still be an accepted tool for gene perturbation (28, 201, 346, 371). The current recommendation to identify a morpholino with minimal off-target effects is to test it in a null mutant background as it should not induce any additional phenotypes (154, 325). Such an approach of course relies on the ability to generate and validate null alleles, for example, by whole-gene dele-

tion (214), without disrupting other genes in the process. Once validated, morpholinos could be used to generate large numbers of embryos with reduced gene function facilitating downstream analyses.

C. Genome Editing

1. Zinc finger nucleases

As concerns about morpholinos’ off-target effects were becoming more acute, sequential advances in reverse genetics—genome editing with zinc finger nucleases (ZFNs), TALENs, and then CRISPRs—revolutionized the zebrafish genetic toolbox (**TABLE 3**). The age of genome editing in zebrafish started with a report that an artificial hybrid assembly of DNA-binding zinc finger modules, each recognizing a specific nucleotide triplet, could be coupled with the sequence-nonspecific nuclease domain of the restriction enzyme FokI to induce double-stranded breaks (DSBs) at specific loci of the zebrafish genome *in vivo* (84). The cellular repair machinery then recognizes the DSB as a substrate for the nonhomologous end joining (NHEJ) DNA repair pathway (400). As NHEJ is an efficient but error-prone system, DSBs often lead to faulty repair and the generation of small local insertions, deletions, or combination indels (84, 242).

A significant drawback in the use of ZFNs is that the zinc finger modules are bulky and interfere with each other, creating difficulty in predicting their binding affinities to a given DNA sequence. Consequently, ZFN assembly and selection demand screening assays to identify working assemblies, which then need to be tested for specificity. While these methods significantly improved over time (229, 332) and mutant alleles for several genes were successfully created using ZFNs in zebrafish, the complicated and time-consuming process of making ZFNs prevented their widespread adoption. Nevertheless, two important lessons were learned: first, modular DNA-binding peptides could be combined with FokI to create sequence-specific DNA scissors. Second, it was possible to induce site-specific DNA mutations in zebrafish through error-prone nonhomologous NHEJ repair of DSBs. Two new technologies incorporating these lessons soon appeared on the horizon.

2. TALENs

Only shortly after the first reports of ZFN activity in zebrafish, a group working on the bacterial plant pathogen *Xanthomonas* was able to break the code for the modular DNA-binding domain of the transcription activator-like effectors (TALEs), a group of type III secreted virulence factors that *Xanthomonas* uses to modulate its hosts’ gene expression program (29). Unlike zinc fingers, each individual TALE module, called a repeat variable diresidue (RVD), recognizes a single nucleotide and does not influence other RVDs’ affinity, allowing highly predictable sequence-spe-

Table 3. Genetic toolbox for the generation of genetically modified zebrafish

Tool	Application	Advantages	Disadvantages	Reference Nos.
Transgenesis tools				
I-SceI transgenesis	Genomic insertion of transgenic constructs	Low bias towards genomic position. Usually single insertion site. Stable expression.	Relatively low efficiency. Possible generation of concatemers.	118, 368
Tol2 transgenesis	Genomic insertion of transgenic constructs	Bias towards open chromatin (see gene trap). High efficiency. No concatemers.	Commonly insertion into several loci.	193, 387
BAC transgenesis	Generation of transgenic lines	Independence from identification of regulatory elements. High chance of endogenous expression pattern.	Very low efficiency in the transgenesis step due to size of the transgene (usually ~150 kb).	40, 108
Gene trap	Generation of transgenic reporter lines; forward genetic screening	Use of endogenous regulatory elements. Fusion proteins. Generation of mutants	Lack of site specificity.	12, 56, 395
Gene knockout tools				
TILLING	Reverse genetics	Can be combined with forward genetic screening. Generates single nucleotide polymorphisms.	Cost intensive and laborious. Possibly outdated with the availability of TALENs and CRISPRs.	177, 254, 375, 424
Zinc finger nucleases	Reverse genetics	Very high sequence specificity due to two proximal recognition sequences.	Time-consuming assembly and testing. Became outdated with the availability of TALENs and CRISPRs.	104
TALEN	Reverse genetics	Very high sequence specificity due to two proximal recognition sequences. Highly efficient. Design tools available.	Somewhat time-consuming assembly (4–5 days). Maintenance of modular assembly library. Risk of library cross-contamination.	22, 43, 83
CRISPR	Reverse genetics	High sequence specificity. Highly efficient. Very simple and rapid assembly (2 days).	Possibly higher risk for off-target lesions compared with TALEN's or Zinc Finger Nucleases.	157, 167, 390
Gene knockdown				
Morpholinos	Transient gene knockdown	Highly established, easy to use, inhibition of target translation or splicing.	Need to validate sequence and amount of morpholino by injecting mutant embryos.	188, 267, 325
CRISPRi	Transient gene knockdown	Easy to use.	Not widely used. Not highly efficient. Possibility of off-target effects.	220, 325
Forward genetic screening				
ENU mutagenesis	Forward genetics/tilling	Mutagenizes during S-phase with no positional bias. Allows the induction of hypomorphic mutations.	Demands mapping efforts to identify the mutation causative of the observed phenotype.	297, 324

Continued

Table 3.—Continued

Tool	Application	Advantages	Disadvantages	Reference Nos.
Insertional mutagenesis	Forward genetics/gene trap	Insertion site relatively easy to identify. Generates reporter lines.	Bias towards euchromatic regions does not allow saturation screens.	265
Genome editing tools				
TALEN	Site-directed genome editing (gene tagging, reporter insertion, generation of large deletions)	Very high sequence specificity.	Somewhat time consuming assembly (4–5 days). Maintenance of modular assembly library. Risk of library cross-contamination.	22, 152
CRISPR	Site-directed genome editing (gene tagging, reporter insertion, generation of large deletions)	High sequence specificity.	Site specificity not always a given due to potential lack of a guide RNA target sequence.	14, 145, 164
Wild-type lines				
AB	Wild-type strain	Highly inbred, cleared from background mutations, widely used.	Low rate of polymorphisms but not isogenic.	
TÜ	Wild-type strain	Highly inbred, widely used. Original reference genome strain.	Medium amount of polymorphisms.	
WIK	Wild-type strain	Highly polymorphic wild strain used for mapping purposes.	Wild strain, normally not used for experimental work other than recombination mapping.	

cific assemblies of RVDs to target a DNA sequence of interest. The knowledge obtained from ZFNs now paved the way for the first highly efficient, yet easy to use genome-editing tool for zebrafish: TALE-nucleases, or TALENs. A TALEN consists of two sequences of RVDs each coupled to a FokI nuclease; each of these RVD “arms” binds to the same target DNA sequence to provide specificity, while FokI induces DSBs.

Major efforts have led to clear and robust design guidelines for TALENs which are available online and do not demand expert knowledge (43, 83, 250, 263) (TABLE 3). Clever and cost efficient molecular cloning and assembly approaches have made it possible to assemble the two DNA-binding arms each consisting of 17–21 nucleotide-specific RVDs and the FokI nuclease rapidly and in large quantities (43, 321). Additionally, several good methods have been established to test TALENs for their efficiency and to genotype embryos with indel mutations (69, 386, 441).

3. CRISPR/Cas9

The discovery of the CRISPR/Cas9 system changed the game once again as it made genome editing and reverse

genetics in zebrafish easier than ever before. Clustered regularly interspaced short palindromic repeats (CRISPRs), together with their associated proteins (Cas), help bacteria recognize and cleave foreign DNA and were therefore adapted to create DNA scissors for any sequence of interest. The general principle of the CRISPR/Cas9 system has already been reviewed many times (82, 158, 231, 405, 423), and thus will not be discussed here in detail. In short, CRISPR/Cas9 is a two-component system that recognizes specific DNA targets by hybridization of a guide RNA (gRNA) and induces DSBs through the nuclease activity of the Cas9 protein.

Unlike ZFNs and TALENs for which creating the DNA-binding part of the scissors is a laborious assembly of zinc fingers or RVDs, target specificity for CRISPR/Cas9 relies on a single gRNA of ~20 nucleotides in length. Therefore, for each desired DNA target, only a sequence of the length of a PCR primer needs to be cloned while the rest of the system remains identical. Due to its remarkable simplicity and adaptability, the CRISPR/Cas9 system rapidly gained popularity and can now be considered the genome-editing tool of choice for most zebrafish researchers. Like TALENs, CRISPRs can be modified not just to cut DNA, but also to

inhibit or activate gene transcription (333). CRISPRs have enabled single laboratories to carry out larger-scale knockouts of several genes at a time, for example, to dissect a genetic pathway (352), to generate mutants from genomic regions of interest (318), or to investigate candidate mutations identified through next-generation sequencing of human disease samples (110, 300). CRISPRs have accelerated existing collaborative efforts to systematically knock out every zebrafish gene (177). Of all the genome-editing technologies, it is the speed and ease of CRISPR that is allowing genome editing at a “high-throughput” pace, a pace that the zebrafish model is well-suited to match.

4. Challenges with CRISPR and other genome editing technologies

Despite CRISPRs’ ease of use, there are still some drawbacks, partially shared by the other genome editing technologies. Cas9 nuclease activity depends on the protospacer adjacent motif (PAM) immediately following the sequence targeted by the guide RNA, thereby often limiting potential target recognition sites. TALENs offer an alternative way to cleave DNA sequences not targetable by CRISPRs, and for this reason, may not become entirely obsolete. This consideration becomes particularly relevant when specific alleles (e.g., phosphorylation sites, DNA binding domains,...) or AT-rich regions need to be targeted for mutagenesis. Statistically, CRISPRs’ short DNA target sequences are present in multiple places in the zebrafish genome, raising concern for off-target mutations (428), whereas TALENs with their two-component recognition offer higher stringency. Finally, NHEJ creates an unpredictable array of alleles that subsequently need to be outbred and screened in what can be a time-consuming process. While NHEJ will likely produce a useful missense or nonsense allele, it is hard to control the kind of mutation generated (see below for the use of oligonucleotides to this end).

Each of these challenges is being addressed. CRISPR design and strategies to mitigate off-target effects are improving (128, 354), and the accepted standards for proving the effect of a mutation now include careful evaluation including RNA and protein expression analyses, as well as functional readouts (FIGURE 4). Generation of specific mutations has also improved and is most frequently achieved by co-injecting a CRISPR with a template oligonucleotide that cells can use to repair the DNA by homologous recombination rather than NHEJ. This template contains a specific mutation of interest, for example, a specific point mutation, or an epitope tag (8). The ability to generate specific mutations is very important for disease modeling, when disease can hinge on a single point mutation, and different mutations even within the same gene can cause different phenotypes.

For CRISPRs as for the other genome-editing technologies, when the sequence of interest allows a choice of where to target the DSB, it is important to target the exon that gives

the best likelihood of causing a null allele. Targeting exon 1 carries the danger that an alternative start codon is used, thereby leading to a functional protein, whereas out-of-frame mutations in later exons can lead to exon-skipping, sometimes preserving protein function. The general recommendation therefore is to consider functional domains and alternative translational start sites, as well as the potential for nonsense-mediated decay of the mutant mRNA (FIGURE 4). Deleting large parts of a gene’s coding sequence is also an option, although one has to consider potential regulatory elements in introns as well as protein(s) and RNA(s) encoded by the reverse strand.

C. Tools for Transgenesis

Key for many zebrafish studies is the ability to generate stable transgenic lines that express genes (e.g., fluorescent marker genes) under specific regulatory elements to perform insertional mutagenesis-based screens, to drive the conditional expression of specific genes, and to design complex transgenic strategies for drug discovery or toxicology. Protocols to stably introduce foreign DNA into the zebrafish genome were pioneered by the Westerfield laboratory in the late 1980s, generating the first transgenic zebrafish that transmitted a bacterial plasmid DNA to their progeny after injecting linearized DNA into the cytoplasm of a fertilized egg (380). Southern Blot analysis revealed that the injection of linear DNA alone was sufficient to achieve high amounts of mosaic somatic transgenesis, and germline transmission was observed in up to 15% of the injected animals (380). The expression of genes from transgenic cassettes, however, turned out not to always occur, probably as a result of rearrangements of the foreign DNA into various types of tandem repeats that potentially induced transcriptional silencing and antisense expression (65). These problems were overcome by the establishment of a protocol that included the flanking of the transgenic cassette with the 18-bp recognition site for the i-Sce meganuclease enzyme. Co-injecting this rare-cutting nuclease with the cassette-carrying plasmid linearizes the circular DNA within the cell and effectively reduces the chance of concatemerization before genome integration. This trick also significantly increased transgenesis efficiency by increasing the integration of low-molecular-weight DNA (392). The use of i-Sce meganuclease has since become widely adopted to generate transgenic zebrafish lines and was further optimized into a standardized protocol (368).

A second method for efficient transgenesis in zebrafish came from the identification and characterization of a hAT transposable element in the Japanese rice fish Medaka (*Oryzias latipes*). Medaka and zebrafish, distant cousins within the teleost branch, do not share the hAT element which made it ideal as a vector to allow transposon-based transgenesis in zebrafish without inducing undesired mobilization of endogenous transposable elements (356). The basic principle

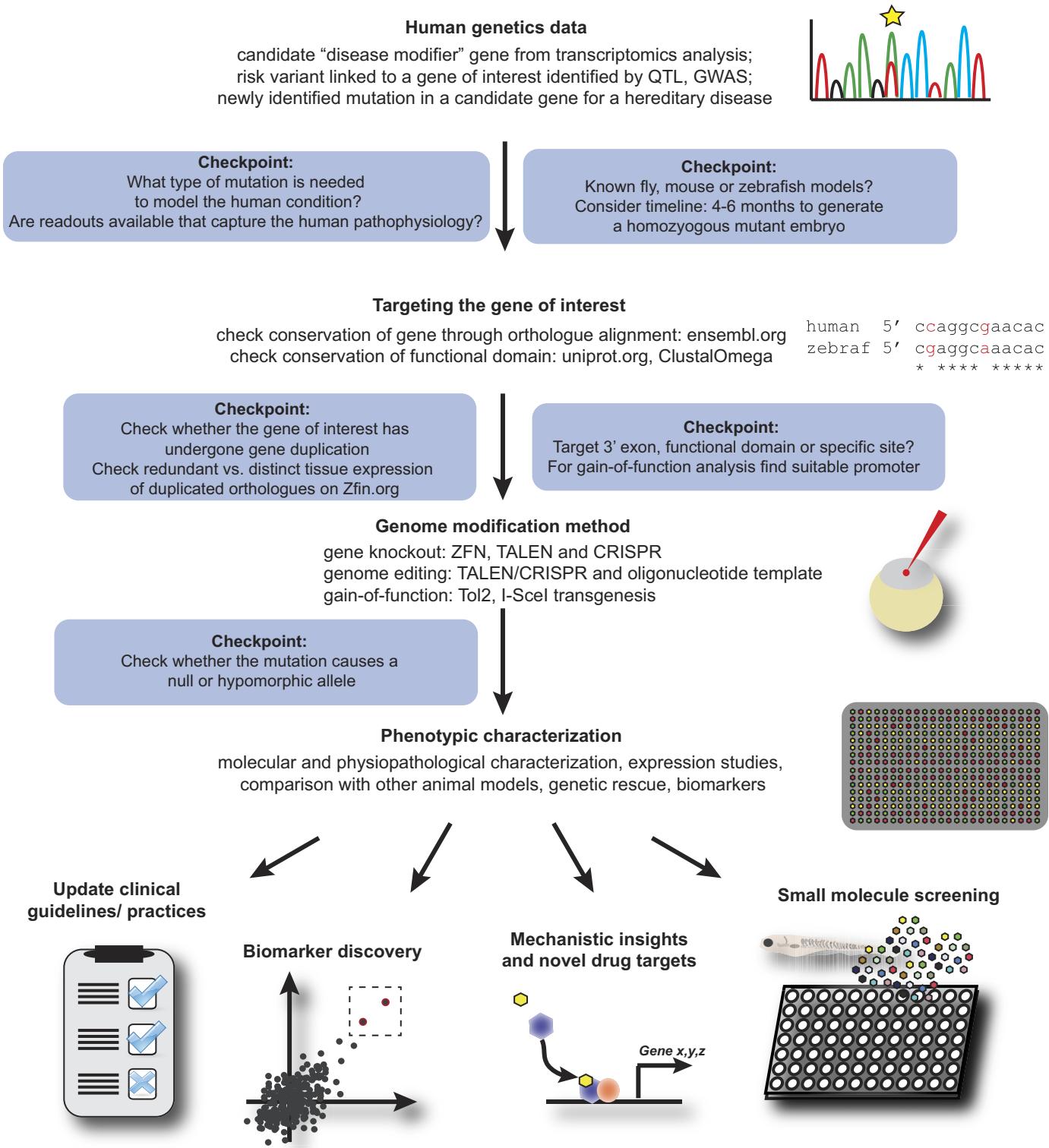


FIGURE 4. Overview of decision roadmap to model genetic disease in zebrafish. Complex and monogenic human diseases can be modeled in zebrafish using different strategies, including genome editing, morpholinos, or transgenic technologies. Several considerations have to be made such as the conservation of genes between zebrafish and human, the timeline of generating genetically modified zebrafish, existence of more suitable animal models, and the availability of meaningful assays to match phenotypic findings to clinical symptoms.

of this system is the separation of the *cis* transposable elements called long terminal repeats (LTRs) from the transposase enzyme. The transposase is transcribed in vitro into mRNA, and the *cis* regulatory elements are used to flank the transgenesis cassette in a circular plasmid. Co-injection leads to very high transgenesis rates, which frequently translate into high germline transmission (175). With the use of this Tol2 system, up to 40% of the injected fish end up having one or more insertions in their germline. Due to the mechanisms of transposon-mediated DNA insertion, plasmid backbones are only rarely integrated using this method. Additionally, Tol2-mediated transgenesis typically results in single insertions per locus, thereby avoiding the formation of concatemers. On the downside, single insertions usually express somewhat lower amounts of the transgene, and transposon-mediated transgenesis has an intrinsic bias for uncondensed chromatin, raising the chances to integrate into gene-harboring loci (191, 210).

With the establishment of meganuclease- and transposon-based protocols, the high transgenesis efficiency has made this technology accessible even to small zebrafish facilities that can quickly develop transgenic lines suited for their research. The limiting factor today is the availability of cloned regulatory elements, enhancers or promoters, to drive time- and tissue-specific expression of genes of interest. The standard way of identifying regulatory elements has been to use PCR to amplify regions located 5' to the transcriptional start site of the gene of interest, but even since the emergence of promoter prediction algorithms, as well as more powerful techniques to identify open chromatin regions such as ATAC-seq, choosing and testing fragments of different sizes can be time-consuming, laborious, and risky. Another strategy to drive gene expression in specific patterns arose from the availability of bacterial artificial chromosome (BAC) libraries. BACs, each containing up to 300 kb of genomic DNA, were originally developed to aid in genome mapping, sequencing, and assembly. In a landmark paper published in 1997, it was shown that the large genomic fragments carried in BACs could be manipulated in bacteria through homologous recombination (431). This finding was adapted to insert a gene of interest at the ATG start codon of an endogenous gene within a BAC, preserving the regulatory sequences without having to know their location, and then integrate the modified BAC into the zebrafish genome. Optimization of BAC transgenesis protocols has led to highly accurate, simple, and efficient methods (40).

III. DISEASE MODELS

"For a large number of problems there will be some animal of choice or a few such animals on which it can be [most] conveniently studied."

—Physiologist August Krogh, 1874-1949

This Nobel Prize winner knew that a good disease model accurately predicts how perturbing a signaling pathway by

genetic, nutritional, or pharmacological means affects human disease outcome. To date, mice and rats have been most commonly used to translate scientific knowledge into clinical predictions, which may then be proven or disproven in human clinical trials. Nevertheless, high-impact rodent studies have had translational success only ~30% of the time (from a median citation count of 889), and only 10% of these successful cases eventually led to a marketed drug (402). The reasons for this low translational efficiency are certainly manyfold and not all solved by using alternative animal models. However, using other model organisms like *Caenorhabditis elegans*, *Drosophila melanogaster*, and zebrafish has helped overcome several methodological shortcomings common with rodent models (342), such as under-powered statistics due to high costs and laborious manipulation (402), inbred strains that do not reflect natural genetic diversity (6), as well as frequent lack of randomization and blinding in research design (23, 106, 236, 402). Zebrafish can fill some of the gaps in current animal experimentation. In this section, we review paradigmatic examples of disease models that have been described in zebrafish and how these models have contributed to our understanding of disease mechanisms (**FIGURE 5 AND TABLES 4-7**).

A. Cardiovascular Disease Models

1. Advantages and disadvantages of the zebrafish cardiovascular system for disease modeling

Zebrafish hold specific anatomic and physiological advantages and disadvantages that are important for cardiovascular disease modeling. Like the human heart, the zebrafish heart develops from a linear heart tube formed by fusion of mesodermal progenitors from a primary heart field (192, 433). The heart tube begins beating via peristaltic motion and loops to form separate atrial and ventricular chambers (317, 373). Cells from the secondary heart field, responsible for the right ventricle and right ventricular outflow tract in mammals, further contribute to ventricular growth (203) (**FIGURE 6**). The atrium and ventricle initially have thin myocardial walls comprising a single cell layer, but soon the ventricle undergoes a process of trabeculation in which clusters of cardiomyocytes of the wall delaminate towards the cardiac lumen to form sheetlike projections, a process which is conserved among higher vertebrates (218). Notably, zebrafish hearts do not septate and do not undergo significant myocardial compaction after trabeculation.

At a border specified by chamber-specific gene expression patterns, atrioventricular and aortic valves form from endocardial cushions by endothelial-to-mesenchymal transition, in a process driven by hemodynamic as well as genetic factors (24, 162, 301, 411). Finally, in juvenile stages ~6 wk post fertilization (wpf), trabecular cardiomyocytes give rise to a new layer of cortical myocardium that surrounds the heart (122). The zebrafish heart is innervated by sympa-

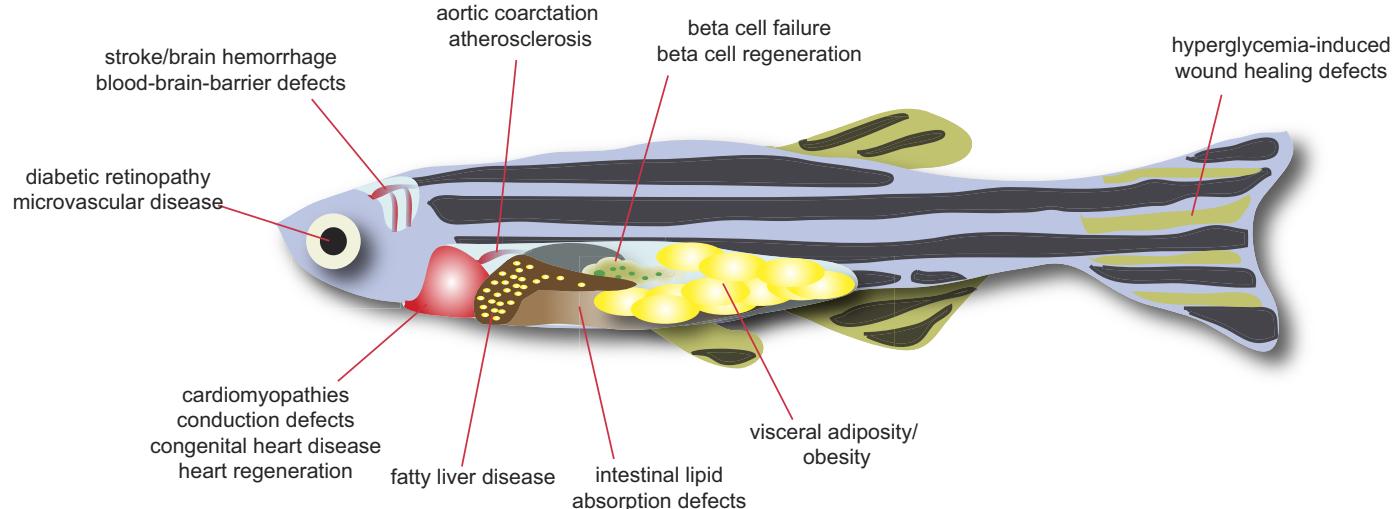


FIGURE 5. Cardiovascular and metabolic diseases studied in zebrafish. Zebrafish have a similar anatomy as that of higher vertebrates. Tissue-intrinsic pathological processes can be studied for a broad range of human cardiovascular and metabolic diseases.

thetic and parasympathetic nerve fibers (377), with cardiac conduction beginning in the sinoatrial node, pausing at the atrioventricular junction, and then speeding through the ventricle resulting in apex-to-base contraction that ejects blood from the heart (47, 246). Unlike murine models, heart rate and EKG parameters representing atrial, atrioventricular, and ventricular depolarization as well as ventricular repolarization are similar to those of humans; for example, heart rate in zebrafish is similar to the 60–100 beats/min in humans, while murine heart rate is 600 beats/min. However, zebrafish lack a specialized His-Purkinje system (9, 246, 289). Zebrafish hearts can also regenerate (179, 230, 307, 413). Physiological assays have been developed for zebrafish hearts including exercise, EKG, telemetry, cardiac output and ejection fraction measurements, and ultrasound, as well as drug screening and drug-inducible gene expression (284, 382, 438). Together, these characteristics make zebrafish an excellent model to study early heart development, arrhythmia, and myocardial function both organ-wide and at high resolution (36, 344, 373).

The zebrafish vascular system also lends itself to disease modeling offering superb visualization of vascular development (FIGURE 6). In a process termed vasculogenesis, endothelial progenitors first migrate to form a cord by 20 hpf (18, 137, 142, 202, 372); by 24 hpf, the single vascular cord has given way to a dorsal aorta and cardinal vein, and circulation begins. Angiogenesis then initiates, first sprouting new intersegmental vessels from the dorsal aorta and then the cardinal vein (137, 141, 202, 360). During vasculogenesis and angiogenesis, arterial and venous fates are specified and aortic arch vessels are defined (138, 141, 202).

Organ-specific vasculature develops at different stages. For example, the blood-brain barrier develops during larval stages, and the zebrafish has been used to study blood-brain

barrier permeability to various drugs and infectious agents (92, 168, 403). Coronary vessels develop much later, several weeks post fertilization, as the zebrafish ventricle has grown significantly since hatching. These vessels arise from angiogenic sprouting and migration of endothelial cells (132, 141). Finally, zebrafish have been used as a highly accessible organism to model vessel injury and inflammation (57), and zebrafish fed a high-fat diet develop lipid depositions in their arteries, creating an *in vivo* model of atherosclerosis important for studying coronary artery disease as will be covered later (97, 98, 376).

2. Cardiomyopathy

One might expect the most highly conserved cardiac functions across vertebrates to be at the cell level. Indeed, some of the first zebrafish heart disease models focused on cell-autonomous cardiomyocyte defects. In 2002, mutations in genes encoding two sarcomeric proteins, Titin and Troponin, were reported to cause embryonic lethal cardiomyopathies (350, 370, 429). Linkage analyses in human families implicated these genes in dilated and hypertrophic cardiomyopathy around the same time (184, 361, 393, 415), followed by several years of dual discovery of various sarcomeric cardiomyopathy mutations in human families and zebrafish forward genetics screens (17, 21, 149, 364, 430). In fact, modern transcriptome analyses show that ~96% of genes associated with human cardiomyopathy are expressed in the zebrafish heart (355).

Reduced cardiac contractility and resultant pericardial edema are clearly scorable in zebrafish larvae, so zebrafish became an easy way to test the pathogenicity of novel variants found in human cardiomyopathy patients, mostly using morpholino gene knockdown. Genes associated with cardiomyopathy soon expanded from those encoding sar-

Table 4. Zebrafish models of cardiovascular disease

Model	Phenotype	Key Findings Reported	Reference Nos.
Cardiomyopathy			
<i>tnnt2a</i> mutant; <i>tnnt2a</i> knockdown	Sarcomere assembly defects	<i>Tnnt2</i> is essential for myocardial sarcomere assembly. Blood flow is essential for ventricular trabeculation. Hemodynamics regulate Notch activity in the endocardium.	73, 301, 350
Inducible myocardial expression <i>PKGB 2057del2</i>	Arrhythmogenic cardiomyopathy (ACM)	Model was used in small compound screen leading to the identification of a drug that reduced ACM in mouse models and human patients iPSC-derived cardiomyocytes.	13
<i>nexilin</i> morphant/overexpression of a pathogenic human <i>NXN</i> allele	Z-disk abnormalities	<i>Nexilin</i> is a component of Z-disks and is essential for Z-disk stability and function. <i>Nexilin</i> mutations associated with dilated cardiomyopathy in humans.	133
<i>erbB2</i> mutant, expression of dn <i>ErbB2</i>	Contractility, trabeculation	<i>ErbB2</i> is a regulator of myofibril localization and bundling. <i>ErbB2</i> regulates trabeculation through cell shape changes. <i>ErbB2</i> is a regulator of cardiomyocyte proliferation.	218, 317, 374
Cardiac expression of an atrial fibrillation associated <i>MYL4</i> p.Glu11Lys allele	Disruption of sarcomeric structure, atrial enlargement, and electrical abnormalities	Pathogenic mutations in structural components of the contractile machinery can cause subtypes of atrial fibrillation.	289
<i>myl3</i> mutant	Cardiac insufficiency, haploinsufficiency, and stress sensitivity	Essential light chain S195 phosphorylation is essential to adapt cardiac contractility under cardiac stress.	337
<i>desma</i> mutant	Desminopathy	Loss of desmin and desmin aggregates cause excitation-contraction coupling defects and reduction of cardiac function. Inhibition of desmin aggregation can restore cardiac function.	311
<i>plcg1</i> mutant	Progressive loss of cardiac contractility	VEGF signaling through PLCgamma1 modulates cardiac contractility.	326
Injection of human amyloid light-chain proteins into circulation	Amyloidosis	Circulating human amyloid light-chain proteins induce cardiomyopathy in zebrafish through cardiomyocyte death in a p38 MAPK dependent pathway. Autophagic flux is critical for the induction of mitochondrial dysfunction and development of amyloid cardiomyopathy.	121, 253
<i>ttna</i> mutants	Low contractility	Analysis of different loss-of-function alleles led to identification of a conserved COOH-terminal internal promoter that is able to restore expression of a truncated, but functional Titin protein. This finding may explain the stronger phenotype of human mutations affecting the COOH-terminal vs the NH ₂ -terminal regions.	444
<i>slc4a1a</i> mutant	Severe anemia, chronic cardiac overload	Chronic cardiac overload induces cardiomegaly through hypertrophy and hyperplasia. Haploinsufficiency of TOR is cardioprotective and attenuates cardiomyopathy in zebrafish. Wnt/β-catenin signaling modulates cardiomyocyte proliferation and hypertrophy.	78, 148, 384
Bolus of doxorubicin and various zebrafish lines derived from an insertional mutagenesis screen, affecting heart function	Endoplasmic reticulum stress and reduced survival	<i>dnajb6b</i> is a susceptibility gene for doxorubicin-induced cardiac myopathy. Ectopic expression of the gene protects against cardiac damage in zebrafish and mouse.	77

meric proteins to transcription factors and mitochondrial, transmembrane, and other regulatory proteins (17, 316, 345, 436) (TABLE 4). Several genes governing cell-cell connections, causing arrhythmogenic and noncompaction cardiomyopathies when defective, were also investigated in zebrafish (133, 186, 235, 257). Morpholinos were used as a quick-and-dirty validation of in silico predictions of mutations causing dilated cardiomyopathy (74). While one might argue that cell-autonomous causes of cardiomyopathy may be just as readily studied in cell culture, zebrafish models provided an organism-wide readout of the various

phenotypes caused; for example, *tax1bp3* causes both cardiomyopathy and cranial abnormalities (345). Also, a whole-organism approach is necessary when the cause of cardiomyopathy is extracardiac, such as with high-output heart failure caused by anemia (384).

More recent studies are moving past pericardial edema phenotyping and morpholino gene knockdown to provide a more sophisticated analysis of mechanisms behind several cardiomyopathies. For example, Zou et al. (444) took advantage of the ease of CRISPR technology in zebrafish to

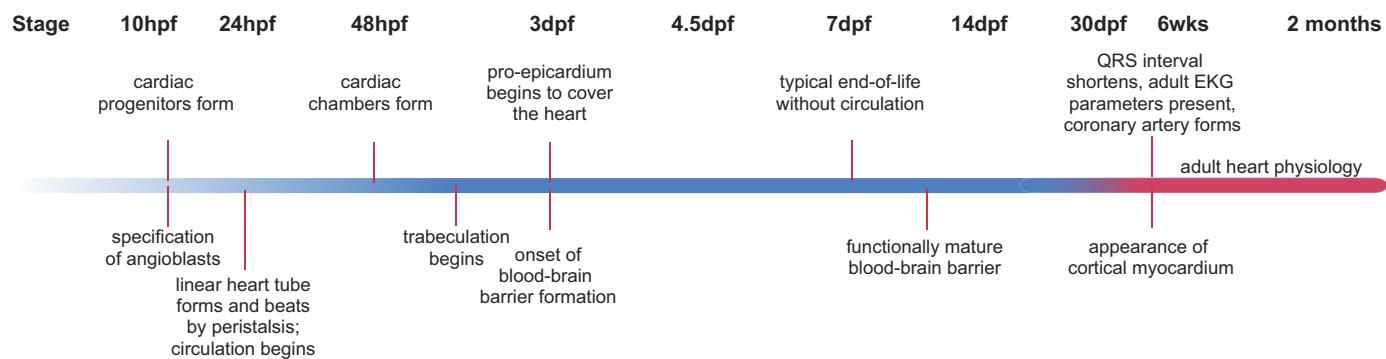


FIGURE 6. Development of cardiovascular physiology. Cardiac peristalsis and blood circulation can be detected by 24 hpf, making the zebrafish a prime model to study early events of cardiovascular development and physiology. The full complement of anatomical and physiological characteristics of an adult heart develops gradually and is considered to be complete by around 6 wk post fertilization. Analogous to cardiac development, the different cell types of the vascular system develop progressively; for example, a fully mature blood-brain barrier is not established before 14 dpf.

create several different *titin* mutations, showing that an internal *titin* promoter may provide partial rescue of some mutant proteins but not others, perhaps explaining some of the range of clinical severity observed in different *titin*-associated cardiomyopathies. Several assays for larval and adult zebrafish have emerged to parse phenotypes more complex than embryonic lethal pericardial edema, and these studies now define a new standard in zebrafish heart failure phenotyping (9, 36, 47, 135, 147, 219, 244, 382). Examples of cardiomyopathies in adult zebrafish include genetically, anemia-, and chemically induced models (13, 76, 78, 384). In a study that exemplifies well the advantage of combining high-resolution imaging with genetic models, Reischauer et al. (317) created a novel transgenic line affinity tagging cardiac actin to show myofibril assembly and architecture during early heart development. Using a dominant-negative *erbb2* transgene, the authors created a model of adult cardiomyopathy in zebrafish. They then used genetic and chemical manipulations of the Erbb2 pathway, associated with chemotherapy-induced cardiomyopathy and of major interest as a therapeutic target, to show that Erbb2 inhibition modulates myofibril organization on a cellular level (317). A recent study by Ding et al. (77) further shows the powerful translational potential towards mammalian cardiomyopathy models: From a transposon-based insertional mutagenesis screen, the authors identified 44 zebrafish lines with cardiac expression of a fluorescent protein indicating genetic lesions at loci that may regulate heart function. Using these lines the authors performed a modifier screen of doxorubicin-induced cardiomyopathy, which pointed to a long isoform of *dnajb6b* that, when disrupted, dramatically increases the susceptibility of adult zebrafish to cardiac damage. In contrast, overexpression of the long isoforms of *dnajb6/DNAJB6* in zebrafish and mice showed to be protective against doxorubicin-induced cardiac myopathy and extended the survival of the animals. Furthermore, ectopic expression of rare *DNAJB6* variants from

human cardiomyopathy patients reduced survival in doxorubicin-treated zebrafish (77).

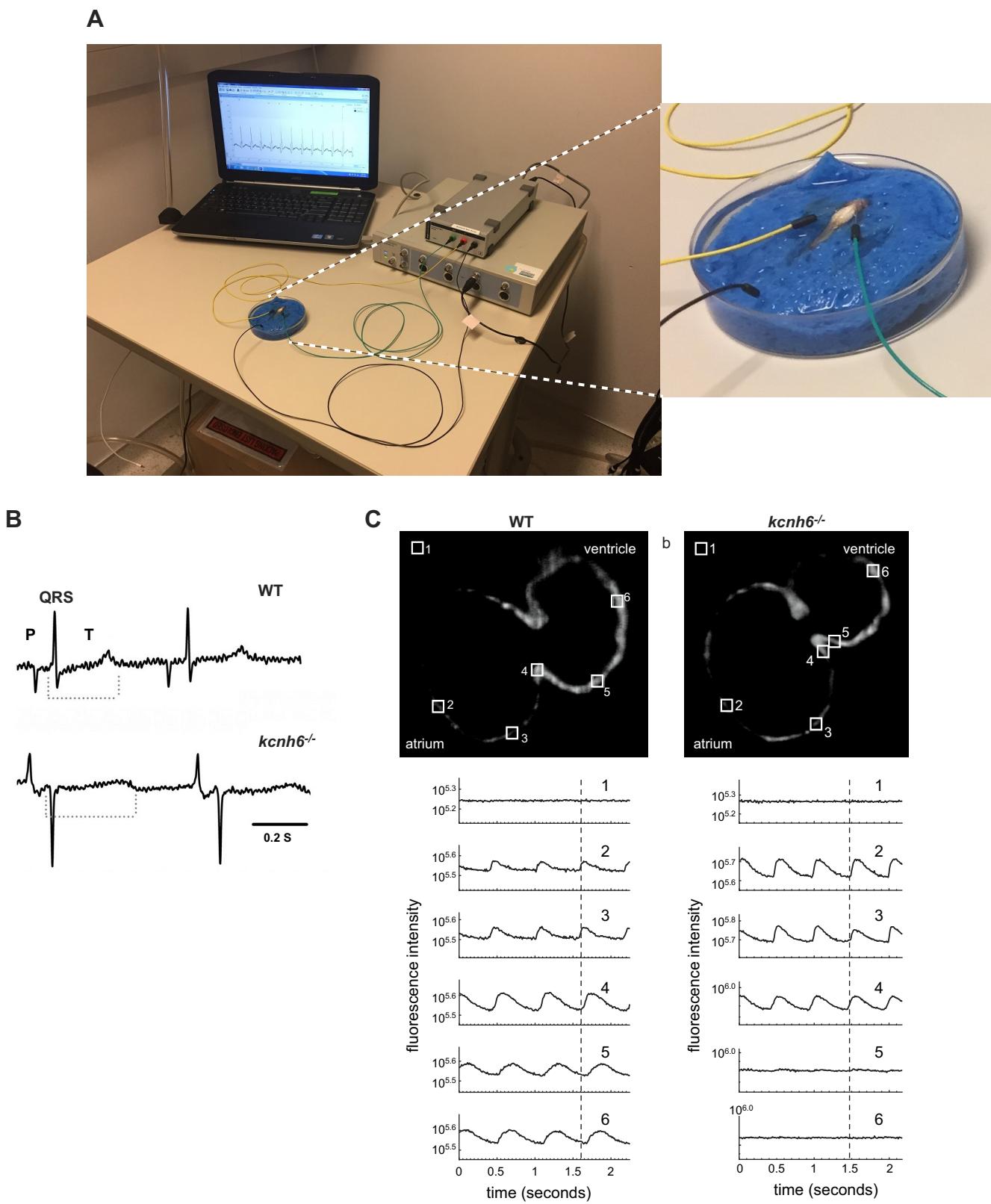
3. Cardiac conduction

Zebrafish models also added new dimensions to the study of cardiac electrophysiology (9, 47, 88, 414) (see FIGURE 7). One of the most powerful examples of this contribution involves the human delayed rectifier potassium channel HERG, defects in which are responsible for Long QT Syndrome Type 2 (LQT2). A range of mutations as well as drugs can prolong the QT interval, creating risk for lethal arrhythmias, and every drug approved by the United States Food and Drug Administration (FDA) requires testing for its effects on the QT interval. Therefore, HERG was already the subject of intense study in *in vitro* and animal models. Mutations in the mouse ortholog, however, had failed to recapitulate human disease phenotypes. Hence, researchers studied LQT2 in larger animal models that were more difficult to maintain and less genetically tractable.

Zebrafish forward genetic screens identified mutants with 2:1 heart block and a silent ventricle; it was soon realized that these different phenotypes all mapped to the *HERG* zebrafish ortholog *kcnh6* (9, 198). The zebrafish LQT2 model proved to hold several advantages in the study of QT prolongation. First, while homozygous mutants exhibited the severe phenotypes mentioned, more careful phenotyping showed that heterozygotes, or wild-type fish treated with QT-prolonging drugs, manifested the same clinical phenotype as humans: a long QT interval on EKG (9, 246). While zebrafish can demonstrate a clinical long QT phenotype, the model can also be designed to show an easily scorable, mechanism-based, binary phenotype of 2:1 heart block—a “high-throughput” phenotype for a clinically relevant disease (196). With the use of zebrafish embryos and this rapid phenotyping, several drug screens were performed validating the zebrafish model’s response to many

known QT-prolonging drugs, finding new genes involved in repolarization, and identifying possible drug therapies for long-QT syndrome (9, 247, 248, 298, 420). The genetic tractability of the zebrafish LQT model, in contrast to the

dog and rabbit models, also allowed a more systematic search for genetic modifiers to the LQT phenotype to help explain the variability in penetrance observed clinically (26, 247).



The *kcnh6* example also illustrates an important lesson about designing phenotypic readouts in zebrafish disease models. An ideal zebrafish model has a documented, clinically relevant phenotype: in this case, long QT on EKG. An alternate, high-throughput phenotype for the same model is also highly desirable, but must be based on the specific disease mechanism at hand, in the way that 2:1 heart block is a mechanistically based high-throughput phenotype for long QT syndrome, while pericardial edema is not a specific phenotype and should not be relied upon.

Second, studying *Kcnh6* and other ion channels in zebrafish has demonstrated a new role for cardiac conduction in structural heart development, at embryonic times when mammalian studies would have simply shown in utero death (9, 45, 46). Similarly, zebrafish were used to study the role of electrical conduction and repolarization during cardiac regeneration, a phenomenon not widely possible in mammalian models (383, 435). As with the cardiomyopathy work, study of conduction in zebrafish first focused on cell-autonomous functions, then cell-cell connections such as gap junctions, while having the advantage of an organ-wide phenotypic readout (353). In studying zebrafish cardiac conduction, researchers have learned how zebrafish hearts are innervated (377), how sinoatrial pacing is set, and how atrioventricular conduction is managed (237, 245, 378). In a remarkable partnership of genetics and imaging, Arrenberg et al. (11) gained optogenetic control of the zebrafish heart (244). Finally, zebrafish has been a useful model for investigating GWAS loci associated with arrhythmia and other novel candidate genes for common arrhythmias found using next generation sequencing (225, 322, 396). In a recent example, whole-exome sequencing identified a cardiac myosin light-chain mutation as a novel cause of heritable atrial fibrillation, and a transgenic zebrafish modeling the same mutation was used to demonstrate that atrial fibrillation in this family was due to atrial myopathy (289). In addition to underscoring the complex relationship between myopathy and arrhythmia, this study highlighted a fact often observed in electrophysiology, where different mutations in the same gene can cause very different phenotypes. As another example, different *HERG* mutations can cause either Long or Short QT Syndrome (9, 37, 134). Therefore, the recent emergence of reverse genetic techniques to efficiently engineer specific mutations, combined with the powerful ability to image and clinically phenotype

arrhythmia, will continue to make the zebrafish a powerful model to investigate conduction disease.

4. Complex congenital cardiovascular disease

Caution must be taken when modeling complex congenital heart disease in fish. Specific phenotypes of human congenital heart diseases, such as septal defects or transposition of great vessels, will be quite different in zebrafish, even when studying orthologous genes. Structural cardiovascular disease is also sometimes part of complex congenital syndromes, as with heterotaxy, diGeorge syndrome, or tetralogy of Fallot.

Even in the case of structural cardiovascular disease, however, the zebrafish model can contribute. Whether genetic or environmental, congenital heart disease has developmental origins. Also, many congenital heart diseases are quite rare (defined by the NIH as affecting fewer than 200,000 people in the United States), greatly limiting clinical research. As a vertebrate model extremely well suited to studying development, the zebrafish is an ideal animal model partner for rare congenital heart disease research. According to the NIH Genetic and Rare Diseases Information Center, roughly one-third of rare diseases affecting the heart have zebrafish models.

Consider Noonan syndrome as an example. Noonan syndrome is an autosomal dominant disease consisting of a range of craniofacial, musculoskeletal, hematologic, and cardiac phenotypes, including stenosis of the pulmonic valve and/or pulmonary arteries. Noonan syndrome is caused by mutations in several different genes, including *PTPN11*, *SOS1*, *RAF1*, and *RIT1* (7, 30, 187, 291, 315). The zebrafish Noonan cardiac phenotype was a heart laterality and looping defect, much more simple and nonspecific than pulmonary stenosis, namely because zebrafish lack a pulmonary valve (30). However, the looping phenotype was scorable and extracardiac phenotypes in zebrafish also echoed Noonan syndrome, adding increased phenotypic specificity to what otherwise is not a highly specific cardiac phenotype on its own. Through work in zebrafish and other models, causative genes were found to all take part in the RAS signaling pathway, redefining Noonan and other related syndromes as RASopathies. Using zebrafish genetics and scorable, if simple, phenotypes to redefine congenital

FIGURE 7. Modeling and phenotyping of cardiac disease. A: schematic of adult zebrafish EKG measurement. An anesthetized zebrafish is positioned ventral side up on a damp sponge; a gill perfusion pump may be used. Needle electrodes are positioned under the skin, and the EKG signal is amplified, filtered, and acquired using standard equipment (246). B: the zebrafish model for Long QT Syndrome Type 2 (LQT2) has a clinically relevant phenotype. Human LQT2 mutations are autosomal dominant and cause a long QT interval on EKG. Analogously, heterozygous *kcnh6* adults also demonstrate prolonged QT interval compared with wild-type siblings. C: homozygous *kcnh6*^{-/-} embryos are amenable to detailed phenotyping, showing absence of ventricular conduction by optical mapping. Wild-type and mutant embryos were crossed into the *Tg(myl7:gCaMP)* fluorescent calcium sensor transgenic background, and imaged using selective plane illumination microscopy. Hearts were stopped to facilitate imaging of conduction. Fluorescence intensity over time was plotted from several regions of interest from the embryos' atrium and ventricle, showing that cyclic fluorescence, and therefore conduction, was absent in the mutant ventricle. This observation was confirmed by patch-clamping the embryonic hearts. [B and C from Annaout et al. (9).]

heart diseases by their molecular mechanisms will improve our understanding of structural heart diseases. Similar principles have been applied to ciliopathies, cohesinopathies, mitral valve prolapse, and vascular diseases including aortic coarctation, arteriovenous malformations, and hereditary hemorrhagic telangiectasia (15, 75, 264, 394, 427). With careful interpretation of analogous phenotypes, and/or use of a supplementary animal model, zebrafish models of complex structural heart disease can improve mechanistic understanding of congenital heart disease and potentially lead to high-throughput screens for therapies for these rare diseases.

5. Cardiovascular regeneration

Throughout this section, the focus has been on whether zebrafish can measure up as a high-throughput, mechanistically informative, and phenotypically sophisticated model for human cardiovascular disease at the cell, tissue, and organ levels. There is one human cardiovascular disease, however, for which researchers are not trying to model zebrafish after human disease, but rather to get human hearts to mimic those of zebrafish. That disease is cardiac injury, which is all too common as a result of myocardial infarction and a leading cause of heart failure. Adult zebrafish hearts regenerate, while adult mammalian hearts do not (307).

Cardiac regeneration is a very active area of zebrafish research and has been discussed in detail in several excellent reviews (103, 328, 389). Notably, researchers have learned that adult zebrafish can regenerate their ventricles without noticeable scar when subjected to surgical amputation or cryoinjury (307). They can also regenerate in response to genetic cardiomyocyte ablation of both atrium and ventricle, even when that ablation is widespread enough to initially cause clinical heart failure (413). Several studies attest to the role of the epicardium as providing mitogenic signals, such as retinoic acid (178); these signals direct subsequent dedifferentiation, proliferation, and redifferentiation of zebrafish cardiomyocytes in response to injury (169). Signaling pathways invoked during development are again seen during cardiac regeneration, such as the Erbb/Neuregulin pathway (113, 174, 179).

Given that even the two-chambered, mononucleate-celled, adult zebrafish myocardium is considered evolutionarily immature in many ways compared with the mammalian heart (306), questions arose as to whether mammals could ever demonstrate cardiac regeneration. In 2011, Porrello et al. (305) reported that neonatal mouse hearts were indeed capable of limited regeneration, finally crossing the divide from fish to mammal. In-depth study of the various pathways allowing cardiac regeneration in response to injury will undoubtedly continue making use of both zebrafish and mammalian systems.

B. Metabolic Disease Models

As with cardiovascular disease, zebrafish have emerged as a popular model for metabolic disease. While the study of zebrafish metabolism is quite valuable, it was slower to develop due to several obstacles that must be recognized and respected to properly model human disease.

1. Advantages and disadvantages of the zebrafish metabolic system for disease modeling

Metabolic disease involves lifestyle, socioeconomic, and behavioral factors unique to humans. Studying energy metabolism in zebrafish also adds technical challenges. For example, assays routine in mammals, like glucose- and insulin-tolerance tests and hormone testing, are cumbersome in zebrafish (87, 185, 234). Zebrafish dietary interventions are difficult to translate to human dietary recommendations due to fundamental differences in macro- and micronutrient requirements (359, 363). Within a tank of zebrafish, it is difficult to track individual food intake; however, food intake is closely linked to growth rate, which in turn influences adipocyte formation, as zebrafish favor somatic growth over body fat deposition through young adulthood (163, 205, 252). This problem becomes evident when rearing a family of wild-type siblings; competition for food, differences in the genetic background, and other factors can lead to large differences in growth rates and adiposity. Temperature also affects growth, metabolic rate, and body fat composition in this poikilothermic animal (216, 408, 409).

The microbiome may also affect metabolism, a factor not controlled for in zebrafish facilities. Compared with conventional or even specific germ-free (SPF) housing of mice, the water circulation carries a large number of potential pathogens. A core microbiome has been reported in zebrafish, and several groups have now begun to explore how naturally or infection-induced differences in the microbiota of the zebrafish intestine alter lipid and glucose homeostasis (20, 94, 95, 144, 303, 313, 314, 426), a concept that is gaining great attention in mammals.

Another angle that remains largely undescribed in zebrafish is the control of systemic metabolism through innervation of glands like the thyroid, chromaffin cells, and the pancreas or that of metabolic organs like enterocytes, the liver, and adipose tissue by sympathetic and parasympathetic inputs. Similarities in the development of the peripheral nervous system as well as the modulation of glucose metabolism by drugs targeting the serotonergic or adrenergic systems suggest the existence of such control mechanisms in zebrafish, but this notion needs to be further explored (91, 124, 224, 258). For these and other reasons, metabolism research in zebrafish has been slow compared with the rapid progress in the cardiovascular field.

Despite these challenges, the fundamental principles of metabolic control appear to be strikingly similar between zebrafish and mammals. Common medicines like the anti-diabetic metformin or the cholesterol-lowering simvastatin have “therapeutic” effects in zebrafish (16, 93, 124), suggesting that new drugs discovered in zebrafish can also benefit humans (226, 443). In this section, we highlight examples that illustrate the utility and value of studying metabolic disease in zebrafish (TABLES 5 AND 6). While zebrafish have been used to study many metabolic diseases including inborn errors of metabolism (238, 391), hyper- and hypothyroidism (153, 437), disorders of the hypothalamus-pituitary-adrenal (HPA) axis (119, 416), dysregulation of the

circadian clock (72, 417), and cancer metabolism (62), we will focus on diabetes, obesity, and dyslipidemia as the three most common metabolic disorders (60, 412a).

2. Development of metabolic control in zebrafish

The control of energy homeostasis through feedback loops across cells and tissues forms the basis of an organism’s survival and function. When this fine-tuned homeostasis is lost, a toxic build-up of carbohydrate, protein, and lipids forms. In diabetes, elevated blood glucose causes both micro- and macrovascular disease (223), which in turn leads to stroke, heart attack, blindness, kidney failure, and neurop-

Table 5. Zebrafish diabetes models

Model	Phenotype	Key Findings Reported	Reference Nos.
Diabetes			
Overexpression of a gene associated with a genetic risk variant for fasting glucose levels: <i>Tg(fabp10a:foxn3,EGFP)</i>	Increased hepatic gluconeogenesis and fasting glucose levels	A SNP in the first exon of <i>FOXN3</i> is associated with human fasting glucose levels. Transgenic experiments in zebrafish confirm this risk variant by showing that Foxn3 overexpression increases hepatic gluconeogenesis and fasting glucose levels.	173
A gene knockout model for monogenic diabetes: <i>pdx1</i> ^{-/-}	Reduced beta cell numbers, decreased insulin and elevated glucose levels; growth retardation	<i>pdx1</i> ^{-/-} show characteristic features of human MODY, type IV. Primary islet forms, but secondary islets do not.	183
Streptozotocin-induced ablation of beta cells	Increased blood glucose levels, followed by beta cell regeneration and recovery to normoglycemia	First study showing that streptozotocin efficiently decreases beta cell mass in adult zebrafish, which is followed by a full recovery within two weeks.	260
Overfeeding a zebrafish line where beta cells are constitutively ablated: <i>Tg(ins:Cre) x Tg(ins:loxP:BFPloxP:DTA)</i>	Rapid proliferation of existing beta cells and increased differentiation from a progenitor pool within the intrapancreatic duct	High nutrient conditions suppress Notch signaling in progenitor cells within the intrapancreatic duct and trigger their differentiation to beta cells in an mTOR dependent mechanism.	273
A zebrafish line with insulin resistance in skeletal muscle: <i>Tg(actc1b:dngf1ra-EGFP)</i>	Skeletal muscle insulin resistance and blunted glucose uptake. Compensatory increase of beta cell numbers	Plasticity of beta cells leading to an increase in beta cell numbers in young animals. Beta cell failure and increased fasting glucose after overnutrition.	228
A model of nonproliferative retinopathy induced by repeated incubation in glucose-enriched water	Prevalent cone photoreceptor dysfunction and impaired vision. Thickening of the retinal vessels in conjunction with the breakdown of interendothelial cell-cell junctions. Absence of neovascularization.	First description of a zebrafish model of diabetic retinopathy (DR). Impairment of cone photoreceptor function independently of neovascularization, the clinical hallmark of DR and primary therapeutic target.	1
Streptozotocin-induced ablation of beta cells	Hyperglycemia followed by return to normoglycemia within 2 wk while wound healing is persistently impaired	Persistent DNA hypomethylation and differences in gene regulation during the wound-healing response in this transient hyperglycemia model. First proof of a “diabetic memory” concept in zebrafish.	286
Streptozotocin-induced ablation of beta cells	Impaired angiogenesis during wound healing in zebrafish that experienced hyperglycemia earlier in life	Transient hyperglycemia impairs angiogenesis during regeneration after return to normoglycemia. Inhibition of poly-ADP ribose polymerase prevents impairment of angiogenesis.	335
A nitroreductase-mediated beta cell ablation system using the prodrug metronidazole: <i>Tg(lnsa:NTR-CFP)</i>	Ablation of beta cells followed by beta cell regeneration and return to normoglycemia within days after ablation	The first model showing efficient regeneration of pancreatic beta cells in zebrafish larvae.	5, 66, 304

Table 6. Zebrafish models of obesity, NAFLD, and atherosclerosis

Model	Phenotype	Key Findings Reported	Reference Nos.
NAFLD			
A genetic model of hepatic steatosis: <i>slc16a6</i> ^{-/-}	Fasting-induced fatty liver disease that is reversed by feeding	<i>slc16a6</i> encodes a ketone body exporter expressed in hepatocytes	159
Gain- and loss-of-function models of the human <i>LXRα</i> and <i>LXRβ</i> orthologs: <i>Tg(factb2:EGFP-nr1h3); Tg(fabp2:EGFP-nr1h3; lxr</i> ^{-/-})	Various effects on lipid metabolism	Enterocyte-specific overexpression of <i>lrx</i> promotes storage of fatty acids in the intestine and protects from hypercholesterolemia and hepatic steatosis	64
Diet-induced obesity	Hepatic steatosis and fibrosis	Ovarian senescence increases the risk for progression to fibrosis in patients with hepatic steatosis	398
<i>ahcy</i> ^{-/-}	Hepatic steatosis and liver degeneration	Mutations in <i>ahcy</i> cause hepatic steatosis in a TNF α -dependent manner	238
Fructose-induced hepatic steatosis	Hepatic lipid accumulation, lipogenesis, inflammation, and ER defects in response to fructose treatment of zebrafish larvae	Rescue of hepatic steatosis by rapamycin treatment	334
Obesity			
Low- and high-density husbandry in combination with caloric restriction or ad libitum feeding	Positive effect of high caloric intake on postembryonic development, metamorphosis, somatic growth, body weight, fat storage, and reproduction	This paper highlights a number of important issues when studying obesity in zebrafish, i.e., juvenile zebrafish are largely resistant against DIO and transform elevated energy intake into an accelerated growth rate. In contrast, middle-aged zebrafish develop severe DIO.	205
Feeding-induced formation of the first adipocytes and visceral fat depots	The first fat-storing cells after the onset of feeding are visceral adipocytes in proximity to the pancreas. Formation of visceral fat depots occurs at late larval stages.	This study is the first to visualize adipocyte formation by making use of nile red staining.	101
<i>plxnd1</i> ^{-/-}	Plxnd1 selectively regulates expansion of visceral adipose tissue.	This paper describes a new role for Plxnd1 in adipose tissue plasticity as well as a number of important assays to monitor adipose tissue formation and metabolic health.	251
<i>lepr</i> ^{-/-}	Absence of obesity, hyperphagia, or infertility. Alterations in glucose homeostasis and beta cell plasticity.	Evolutionary diversification of obesogenic and glucose regulatory functions of leptin between fish and mammals.	243
Overexpression of <i>agrp</i>	Increased obesity and somatic growth	This study reports the first teleost obesity model in response to modulation of central mechanisms of fat storage.	367
Atherosclerosis/dyslipidemia			
High-cholesterol diet	Vascular lipid accumulation; accumulation of myeloid cells in the vascular wall. Vascular leakage in response to chronic high-cholesterol diet	First model of high-cholesterol diet-induced atherosclerosis in zebrafish; regulation of lipid uptake into macrophages by Toll-like receptor 4.	376
High-cholesterol diet	Vascular lipid accumulation	Visualization of an immunogenic atherosclerotic plaque in vivo using an EGFP-labeled single-chain human monoclonal antibody.	96
<i>apoc2</i> mutant	Hypertriglyceridemia, chylomicronemia	<i>apoc2</i> deficiency leads to accumulation of lipid-laden macrophages in blood vessels	217

athy (60, 223, 262, 410). Due to its multi-organ effects, metabolic control must be understood at a system-wide, organismal level to develop new therapies.

Just like body plan formation, metabolic homeostasis is an important intrinsic feature of development. Evolving dynamics of metabolic feedback circuitry can be observed

alongside the zebrafish's anatomical development: organs such as the thyroid and the endocrine pancreas have developed within 48 hpf and actively secrete hormones that are critical for energy homeostasis (153, 171, 234, 283, 287). Development of metabolic homeostasis begins with establishment of glucose regulation. Glucose levels follow well-defined patterns, demonstrating two distinct peaks at 24

hpf and 5 dpf (**FIGURE 8**). Interestingly, both peaks are followed by increased beta cell proliferation, suggesting a delay of homeostatic feedback signals or even a developmental role for glucose in triggering beta cell proliferation (124, 171, 397). Between 48 hpf and 5 dpf, the whole-organism glucose concentration drops, followed by a second decline when the yolk has been consumed and the zebrafish transitions to a fasting state. Like mammals, zebrafish can adapt to low energy supply by shutting down energy consumption in most tissues, while activating a gluconeogenic program in the liver (124, 171). Early glucose dynamics are counterregulated by insulin; impaired beta cell development or ablation of beta cells as early as 2 dpf leads to a steep rise in glucose levels that recovers when beta cells are allowed to regenerate (5, 183).

Recently, Karanth et al. (173) showed that monitoring gluconeogenesis in zebrafish can be used to probe genetic variants. A SNP located within the first intron of *FOXN3* had been identified in genome-wide association studies as a risk variant for elevated fasting blood glucose levels. Overexpression and loss-of-function studies in zebrafish revealed that *FOXN3*/*foxn3* stimulates hepatic gluconeogenesis in larval and adult zebrafish consistent with the higher mRNA expression of *FOXN3* in human carriers of the risk allele.

3. Yolk-feeding to fasting transition as an experimental advantage

The yolk-feeding to fasting transition provides an easy experimental system to study metabolic adaptations to en-

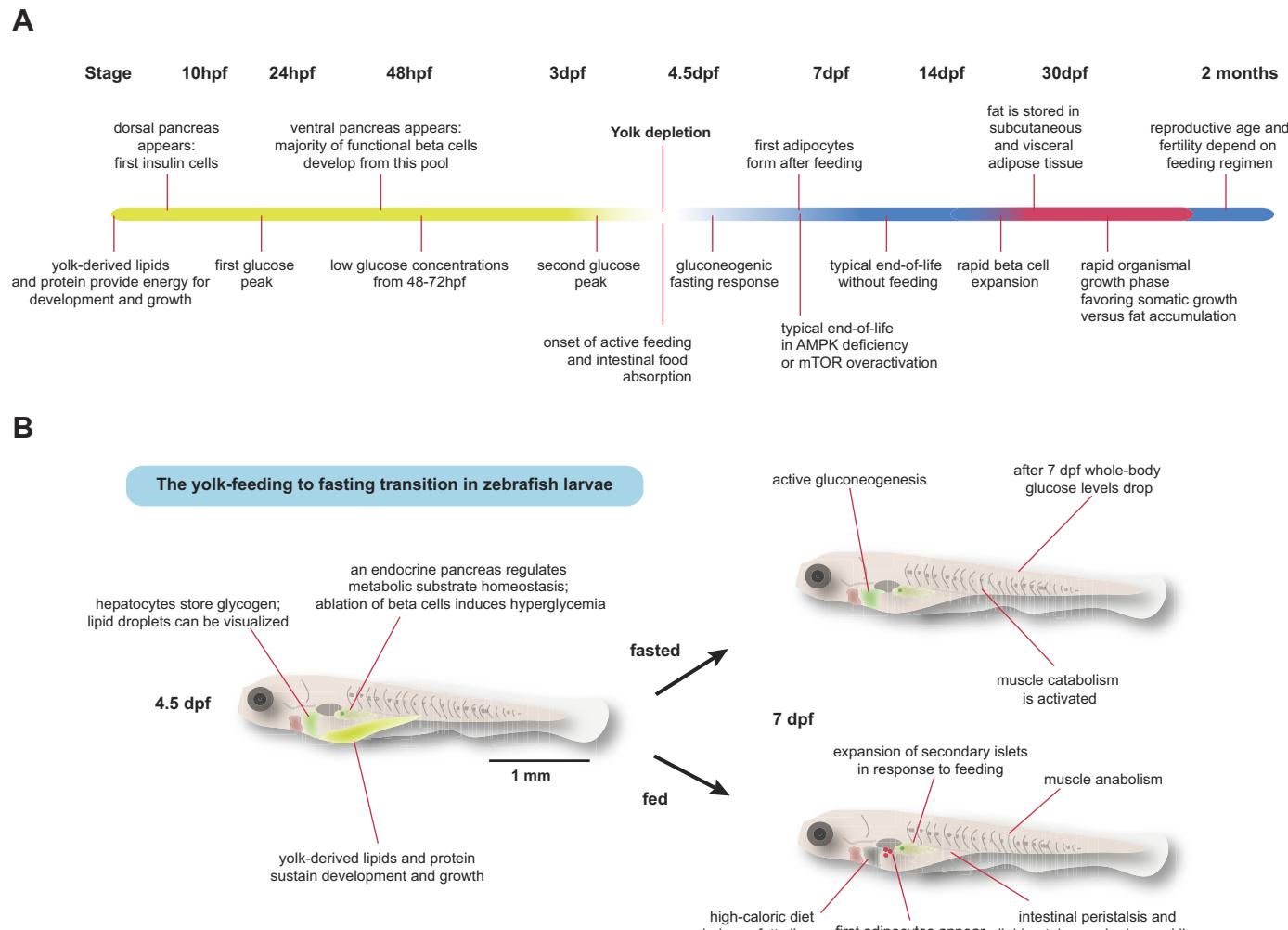


FIGURE 8. Development of metabolic regulation in zebrafish. **A:** timeline of metabolic characteristics throughout development. Glucose levels are highly dynamic during development and are regulated at early stages by endogenous glucose production, glucose utilization, as well as hormones, as in mammalian glucose regulation. Lipid homeostasis is determined at early stages by release of stored lipids within the yolk sac. The first adipose cells appear after feeding commences, and larger fat depots are not formed before ~30 dpf. **B:** energy substrates in the yolk sustain growth and metabolism, but are depleted around 4.5 dpf. If larvae are not fed by this time, they switch to a fasted state, and metabolic adaptations set in that are reminiscent to mammalian energy-sensing mechanisms. This defined yolk-feeding to fasting transition can be leveraged to monitor energy-sensing mechanisms. In addition, feeding of zebrafish larvae can be used to monitor adipocyte formation or intestinal lipid processing [Modified from Schlegel and Gut (341).]

ergy scarcity in thousands of animals and without external feeding bias. A study that exemplifies well the power of this approach screened hundreds of zebrafish families from an ENU mutagenesis screen for animals that develop fatty liver. The group identified a mutant, *red moon*, which is characterized by hepatic steatosis during late larval stages. Mechanistic analyses found that the mutated gene encodes *Slc16a6a*, a monocarboxylate transporter which helps export ketone bodies from the liver to supply energy to tissues during fasting (159). Chronically fasted *slc16a6a*^{-/-} mutants die from lack of sufficient ketone body supply to peripheral tissues. Importantly, hepatic lipid accumulation in *slc16a6a*^{-/-} mutants is reversible with feeding, when endogenous ketone body production is suppressed.

Cellular energy sensing during the transition to fasting is a key feature of cells to adapt metabolism to nutrient deprivation. Of the four major energy-sensing complexes, insulin/insulin-like growth factor 1 (IGF1), mTOR, and AMPK have been found to have well-conserved functions. Disruption of the major energy sensor complex AMPK in zebrafish that lack the upstream kinase Lkb1 die prematurely at 7 dpf, 2 days after the yolk has been consumed (401). A similar phenotype is observed in *tsc2*^{-/-} animals, which are characterized by a constitutive activation of mTOR signaling. These animals show cellular hyperplasia and, similar to *lkb1*^{-/-} mutants, premature death at 7 dpf. Consistent with conserved mechanisms of mTOR signaling, the *tsc2* mutant phenotype can be reversed by treating with rapamycin, an inhibitor of the mTOR complex downstream of Tsc2 (180). The fourth major class of energy-sensing complexes, sirtuins, which are NAD⁺-dependent deacetylases, are to date not well studied in zebrafish (125, 155). Morpholino knockdown of the zebrafish *sirt1* ortholog impairs endothelial sprouting and vascular morphogenesis (308). However, stable loss-of-function models and proof for a role of sirtuins in zebrafish metabolic control are still lacking, despite evolutionary conservation with 1-to-1 orthologs for each of the seven sirtuin genes.

The yolk-feeding to fasting transition can also be leveraged for drug screening. A small-molecule screen for endogenous induction of pyruvate carboxykinase 1 (*pck1*) expression during the transition identified compounds that modulate organismal fasting metabolism. The screen of ~3,000 compounds revealed that several ligands of Tspo, a protein located to the outer mitochondrial membrane, are activators of hepatic gluconeogenesis while potently suppressing whole-body glucose levels. These ligands activate a Ppara-mediated gene signature resembling a canonical mammalian fasting response. These compounds were also able to protect mice with diet-induced obesity against hepatic steatosis and improve glucose handling (123, 124).

4. Adipose tissue plasticity and obesity

Obesity results from incrementally storing large amounts of dietary triglycerides in adipose tissue. Although obesity is now catalogued as a disease in itself, an individual's propensity to develop diabetes, non-alcoholic fatty liver disease (NAFLD), atherosclerosis, and their fatal complications varies dramatically based on genetic background, gender, lifestyle, and environmental factors. The existence of "healthy obese" individuals is debated, but reflects the idea that a subset of individuals are considered healthy with respect to cardiovascular parameters, insulin resistance, glucose handling, and blood lipid profiles despite profound adiposity. A high capacity to store neutral lipids and avoid systemic inflammation rather than exposing peripheral tissues to triglycerides, cholesterol, and fatty acids is believed to have a major influence towards preventing obesity-associated complications (166, 194, 381, 421). As such, identifying factors regulating adipose tissue development and plasticity is key to understanding why some individuals escape the detrimental consequences of obesity. Genetic and nutritional factors that regulate the plasticity and expansion of adipose tissues, mechanisms of lipid absorption, packaging and transport, as well as inflammatory responses to aggressive lipid moieties are starting to be investigated in zebrafish (3, 339, 341, 351).

Adipose development in zebrafish follows the same basic principles as in mammals. Larvae soaked in the neutral lipid dye nile red first show adipocytes shortly after they commence feeding around 7 dpf, whereas no adipocytes develop in food-deprived animals (101). The yolk sac stores and synthesizes lipids during the first 4 days of development (105), but its anatomical structure and packaging of energy substrates has little resemblance to that of human adipose tissue. Subcutaneous and visceral fat pads that anatomically match human adipose tissue first appear around 30 dpf when the energy is no longer completely converted into somatic growth (101, 163, 205, 252) (FIGURE 8). This late appearance of adipocyte tissue weakens many of the advantages of the zebrafish model such as easy *in vivo* imaging or rapid drug testing in multi-well format. Along these lines, attempts to identify a promoter that selectively labels zebrafish adipocytes for lineage tracing or spatio-temporal control of gene expression have been unsuccessful thus far. Several studies have shown that lipid uptake, metabolism, and storage are tightly regulated in zebrafish and that chronic overfeeding with lipid-rich diets ultimately leads to insulin resistance (101, 209, 228, 252), NAFLD (64), obesity (205, 228, 252, 285), and atherosclerosis (97, 98, 376) (TABLES 5 AND 6).

Genetic loss- and gain-of-function studies have also shown that many genetic factors underlying adiposity in zebrafish are similar to those in mammals (51, 241, 367). However, knockout of one of the most extensively studied signaling pathways that determines body fat mass, leptin signaling,

fails to show a conserved role. Human patients with mutations affecting leptin signaling show excessive eating patterns and are morbidly obese and less fertile compared with healthy individuals. Mouse models show similar phenotypes; leptin is highly expressed in mammalian adipocytes, and its secretion is stimulated by insulin after food intake (107). In contrast, zebrafish that lack the leptin receptor *lepr* show normal growth, fat mass, feeding behavior, and fertility (243). Expression of the *lep* gene in zebrafish is nearly absent in fat, whereas mRNA levels are increased during fasting in the liver.

Instead, leptin signaling in zebrafish appears to affect glucose homeostasis. In depth phenotyping of zebrafish lacking *lepr*^{-/-} or *lepa*^{-/-} orthologs revealed increased insulin sensitivity and glucose clearance after a glucose tolerance test in adult zebrafish. Larval zebrafish showed a higher beta cell number while having a reduced compensatory increase in the number of beta cells in response to overfeeding. Lastly, wound healing after fin clipping was slower in *lepr*^{-/-} animals compared with wild-type. This recent study points out important molecular differences between zebrafish and mammalian adipose tissues, highlighting the possibility of dissecting the divergent roles of leptin signaling in glucose homeostasis without the confounding effect of excessive fat mass (243).

Zebrafish work has already led to truly novel insights in obesity research. For example, recent work has revealed a mechanism for *PLXND1*, a gene associated in population genetics studies with differences in human hip-to-waist fat distribution as well as an increased risk for type 2 diabetes mellitus (T2DM) (358). The mouse knockout of *Plexin D1* causes embryonic lethality before visceral fat development (251), but *plxnd1*^{-/-} zebrafish are amenable to study. Interestingly, visceral fat depots in the zebrafish mutant are hyperplastic with a reduced capacity to store lipids (251). In contrast, subcutaneous fat appears unaffected, suggesting a role for *plxnd1* in adipose tissue distribution. In high-fat diet (HFD) conditions, the visceral fat tissue was unable to expand thereby forcing the redistribution of excess lipids to subcutaneous depots. The underlying mechanism was shown to depend on the remodeling of the surrounding extracellular matrix. In addition to revealing novel *plxnd1* functions, this work describes an interesting set of *in vivo* and *ex vivo* adipose tissue imaging techniques that should be of great use for similar investigations in the future (251, 252).

Despite the absence of adipose tissue during early embryonic and larval zebrafish development, other aspects of lipid metabolism can be studied at these early stages; for example, the gastrointestinal system of zebrafish is anatomically and functionally closely related to that of mammals (3, 99, 341). Feeding of fluorescently labeled lipid moieties to zebrafish larvae has been leveraged to visualize lipid absorp-

tion and processing in enterocytes. A forward genetic screen using this assay, for example, identified the *fat-free* mutant, which showed unaltered intestinal morphology despite reduced phospholipid and cholesterol processing (99). It was later shown that *fat-free* encodes Ang2, a conserved subunit of the Golgi-associated retrograde protein (GARP) complex, which regulates lysosomal enzyme sorting, endosomal cholesterol trafficking, and autophagy (146, 299).

5. Non-alcoholic fatty liver disease

NAFLD is the most frequent lipid storage disorder in the liver independent of alcohol consumption and is driven mainly by high-fat diets and carbohydrate-rich foods (213). It affects over 30% of the general population and an even higher percentage of obese individuals and is considered a complication of obesity. It is reversible through dieting, but if untreated predisposes to a sequence of severe liver pathologies: non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma (221). Which genetic factors drive the progression from benign NALFD towards inflammation, fibrosis, and ultimately malignant liver cancer are poorly understood (59).

Studying NAFLD in zebrafish has the advantage that steatosis can be visualized in larvae by whole-mount Oil Red O staining for neutral lipids (340, 343). With the onset of feeding at 4 dpf, zebrafish can be fed lipid-rich diets such as an emulsion of egg yolk (209) or *Artemia* brine shrimp (285). Alternatively, ketogenic or fructose-based diets have been used to trigger steatosis (172, 334). The larval liver is big enough to be dissected with relative ease, and transcriptomic, histological, and biochemical analyses can be performed on these samples (124, 172). A broad range of transgenic lines exist to visualize different hepatic cell types, including hepatocytes, biliary cells, endothelial cells, and stellate cells (331, 336, 434). Adult zebrafish can be chronically exposed to defined diets and lipid content of the liver measured by conventional enzymatic assays or metabolomics (159, 172). Radiolabeled tracers can be used to follow carbon flux into different biosynthetic pathways (172).

The first zebrafish models of NAFLD were again found in large-scale forward genetics screens, suggesting that systematic screening for NAFLD-causing mutations and investigating their role in disease progression can reveal important insights into disease mechanisms (159, 238, 279, 330) (TABLE 6). A single recent ENU mutagenesis screen alone identified 19 new mutant lines that are characterized by hepatomegaly, with most of them displaying prominent neutral lipid staining. Many of these mutants exhibit unique histological pathological features ranging from mild micro- or macrovesicular steatosis to severe signs of NASH characterized by ballooning and necrosis of hepatocytes (181). Cloning the genes affected by these mutations will likely contribute important insights into the development of chronic liver disease.

6. Dyslipidemia and atherosclerosis

Studying atherosclerosis in zebrafish has similar technical advantages as studying NAFLD including the possibility to stain lipid deposits within blood vessels *in vivo*. A set of groundbreaking studies by the Miller laboratory showed that unlike in rodents, wild-type zebrafish that are chronically exposed to high-cholesterol diets have a propensity to develop histopathological changes similar to those seen in human atherosclerosis (96–98, 376). [Murine atherosclerosis studies therefore rely on a sensitized background such as *ApoE*^{-/-} animals (35).] An evolutionary basis for this phenomenon is that the ortholog of the human cholestryl ester transfer protein (CETP) is conserved in zebrafish, while it has disappeared from rodent genomes. Therefore, as in humans, cholestryl esters in zebrafish are diverted from protective HDL cholesterol particles to “bad” LDL, thereby increasing the susceptibility for atherogenic events. To exploit the advantages of zebrafish for *in vivo* imaging, the Miller group (96) also showed that oxidation of LDL by malondialdehyde can be visualized using single-chain monoclonal antibodies linked to GFP. Furthermore, recruitment of macrophages to vascular lesions can be monitored and quantified to find new treatment strategies.

Liver X receptor (encoded by *LXRA* and *LXRB* in humans) is a master regulator of cholesterol homeostasis in mammals (33) which appears to have functionally conserved roles in zebrafish. In contrast to mice, however, only one *lxr* ortholog exists in zebrafish (encoded by *lxra/nr1h3*) allowing one to capture its physiological roles with relatively straightforward gain- and loss-of-function experiments. *lxra*^{-/-} knockout zebrafish are viable, but mirror the cholesterol intolerance of *Lxra*^{-/-}, *Lxrb*^{-/-} double knockout mice which show excessive cholesterol levels in the liver and blood after a chronic HFD. Through over-expression experiments, the authors also showed that increased Lxr function specifically in enterocytes could cause the opposite effect, i.e., in response to HFD the accumulation of cholesterol in the blood and liver was strongly slowed down (64). Mechanistically, it was found that Lxr activates an acyl-CoA synthetase (encoded by *acsL3a*) that metabolizes absorbed lipids and targets them for storage in lipid droplets instead of a quick release into the blood. Clinically, this discovery is of importance as several studies indicate that postprandial increases of cholesterol drive atherosclerosis. Postprandial dynamics of cholesterol, however, is a process that is not particularly well controlled by statins (which inhibit the de novo synthesis of cholesterol in the liver). Targeting the Lxr-AcsL3a axis may therefore be an interesting strategy to slow the release of absorbed dietary lipids into the circulation, thereby acting synergistically with statin treatment (64, 341).

7. Regenerative approaches to diabetes

Zebrafish have a remarkable plasticity of beta cell mass, in response to both injury and metabolic challenges (5, 209, 228, 260). Dissection of the genetic cues that determine the development, maturation, and plasticity of functional beta cells has been a primary focus in zebrafish research and is starting to contribute important insights for beta cell regeneration and replacement therapies. Several distinct approaches are being probed in this context, ranging from the differentiation of mature beta cells from induced pluripotent stem cells *in vitro* to coaxing remaining beta cells, progenitor pools, or even non-beta cells into restoring adequate numbers of functional beta cells *in vivo* (5, 68, 208, 406, 439, 440).

Anatomically, zebrafish are well suited for this purpose because each progenitor and cell type within the islet can be visualized and monitored over time *in vivo* using fluorescent reporter proteins until the formation of a mature pancreas throughout embryonic, larval, juvenile, and adult stages (70, 80, 143, 261, 272, 273, 432). Pancreatic islets in zebrafish include a single primary islet in the head of the pancreas as well as secondary islets dispersed throughout the organ (100, 296).

The first studies suggesting that zebrafish could be used to dissect the genetic network governing endocrine pancreas development came once again from forward genetic screens (292, 385). An insertional mutagenesis screen identified a line with a mutation in the homeobox gene *vhnf1*, which fails to express normal levels of *pdx1*, a transcription factor gene necessary for the induction of the insulin gene (385). The disease relevance of these findings became clear as lesions in these genes are known to cause a subtype of monogenic diabetes: mature-onset diabetes of the young (MODY) type V for *VHNF1* and type IV for *PDX1*. Indeed, it was later shown that deletion of *pdx1* in zebrafish causes a reduction in beta cells, decreased insulin levels, and disrupted glucose homeostasis (183) (TABLE 5). To date, a large number of studies have shown that genes specifying hepatopancreatic cell lineages have highly conserved functions (52–54, 79, 100, 117, 143, 232, 233, 269, 283, 369).

Drug screens are now being carried out to find small molecules that induce or block different steps of endocrine lineage specification and maturation. These molecules may ultimately be used to improve the efficiency of the generation of mature beta cells from iPS cells *in vitro* or to activate progenitor pools that can generate new beta cells *in vivo* (68). A screen that exemplifies this approach used a double-transgenic line *Tg(Tp1:hmgb1-mCherry)*, *Tg(pax6b:GFP)* to visualize *pax6b*-positive progenitor cells within the intra-pancreatic duct (327). *Tg(Tp1:hmgb1-mCherry)* animals carry a Notch activity reporter that marks cells of the intra-pancreatic duct, and upon suppression of Notch signaling, these progenitor cells become activated to become beta cells

in a process similar to the differentiation of progenitor cells in the mammalian pancreatic duct (266). This line was subsequently screened for compounds that induce the occurrence of secondary islets as marked by *pax6b*:GFP expression at 5 dpf (296, 327), a stage when no secondary islets have formed during normal development.

Zebrafish have also been used to systematically interrogate pathways required to enhance the regeneration of beta cells after tissue injury using a chemical genetics approach. Beta cell ablation studies have shown that normal numbers of beta cells recover within days even after a near-total ablation of beta cells (**TABLE 5**) (66, 260, 304). With the use of this ablation paradigm, a screen of over 7000 small molecules revealed that the largest group of hit compounds restoring beta cell mass activates adenosine signaling and triggers compensatory proliferation of the regenerating beta cells. The strongest hit, the pan-adenosine receptor agonist NECA, was also able to increase proliferation of beta cells in mice subjected to streptozotocin-induced beta cell ablation, and to restore glucose homeostasis (5). In a follow-up study, it was shown that the effects on beta cell proliferation were mediated by the adenosine receptor A2a, which in fact is specifically implicated during compensatory beta cell proliferation, including post-injury as well as during pregnancy (347).

Lineage tracing and beta cell ablation tools have been used to study aspects of beta cell plasticity in response to metabolic challenges, a phenotype that has been observed in obese individuals, but is mechanistically poorly understood (27). Similar to what is observed in human obesity, zebrafish beta cells are transiently able to adapt to the increased demand for insulin by increasing overall beta cell mass. Overnutrition of zebrafish larvae and juveniles leads to a suppression of Notch signaling in endocrine progenitors which causes their differentiation into beta cells. This plasticity was further shown to depend on mTOR-induced suppression of Notch signaling (273). Beta cell plasticity has also been shown to occur in adult zebrafish in response to excess food intake. Lipid-rich diets as well as glucose trigger compensatory beta cell expansion based on distinct mechanisms, i.e., whereas the response to high glucose levels requires active mTOR signaling, the response to lipids depends on insulin/IGF1 signaling (209, 227). The newly formed beta cells could be further characterized by two subpopulations of postmitotic precursor cells positive for *nkx2.2* or *mnx1* expression.

In a recent report, zebrafish studies contributed to the identification of artemisinins as small molecules that in response to beta cell ablation repress alpha cell identity and lead to enhanced beta cell regeneration as well as improved glucose control (208). On the basis of this and many other examples, the vast collection of transgenic reporter lines and islet-cell ablation tools hold promise to systematically inter-

rogate all steps of beta cell development and plasticity in healthy and stressed conditions. A close collaboration with groups specialized in stem cell biology and regenerative medicine will be necessary to support the best therapeutic strategies with knowledge collected from zebrafish studies.

IV. DRUG DISCOVERY USING ZEBRAFISH

“A very interesting set of compounds that were waiting for the right disease.”

—Doctor Jerome Horowitz, 1919-2012

That is how researcher Jerome Horowitz described the dideoxythymidine compounds he developed in the 1960s, hoping to cure cancer. Instead, these drugs—the most famous of them being zidovudine, or AZT—became the first to successfully fight HIV infections, decades after they had been forgotten as a failed cancer treatment. Drug discovery has powered some of the most revolutionary advances in modern medicine. Zebrafish are increasingly used both in the academic and pharmaceutical worlds to address major challenges for drug discovery: to identify new drug-target interactions, to match existing drugs to new uses, and to evaluate for toxicity and efficacy *in vivo*, all at a scale that keeps up with medical need.

Drug discovery follows two principal strategies: target-based and phenotype-based screening (89). Target-focused drug discovery has become the center of investment in today’s pharmaceutical industry due to the ultra-high-throughput screening capabilities on purified protein target assays (388). This target-focused approach follows a defined sequence of steps: target discovery, high-throughput screening *in vitro*, *in silico*-assisted toxicology studies in cells and preclinical models, and ultimately clinical trials (255) (**FIGURE 9**). An increasingly applied alternative strategy is phenotypic screening which aims to identify compounds that exert a desired physiological effect without a priori knowledge of the target (90). The use of phenotypic screens in cellular systems such as immortalized cell lines, primary tissues, and induced pluripotent stem cells have evolved rapidly alongside emerging technologies in robotics, high-content imaging, image processing, and automated analysis (31). However, both target-focused and phenotypic strategies still face the challenge of a lengthy development phase (89) (**FIGURE 9**).

Over 80 chemical screens over the past 16 yr attest to the zebrafish’s utility in whole-organism, phenotype-based drug discovery (226, 319). Zebrafish cell populations or molecular pathways can be monitored at stages when zebrafish larvae are still small enough to be placed in 96-well plates. Simple addition of chemicals to larval medium then allows one to screen large numbers of chemicals by the phenotypic response of the larvae in each well (131). The similarities of zebrafish and human genes suggest that many targets are in principle conserved (156, 226) (**TABLE 2**).

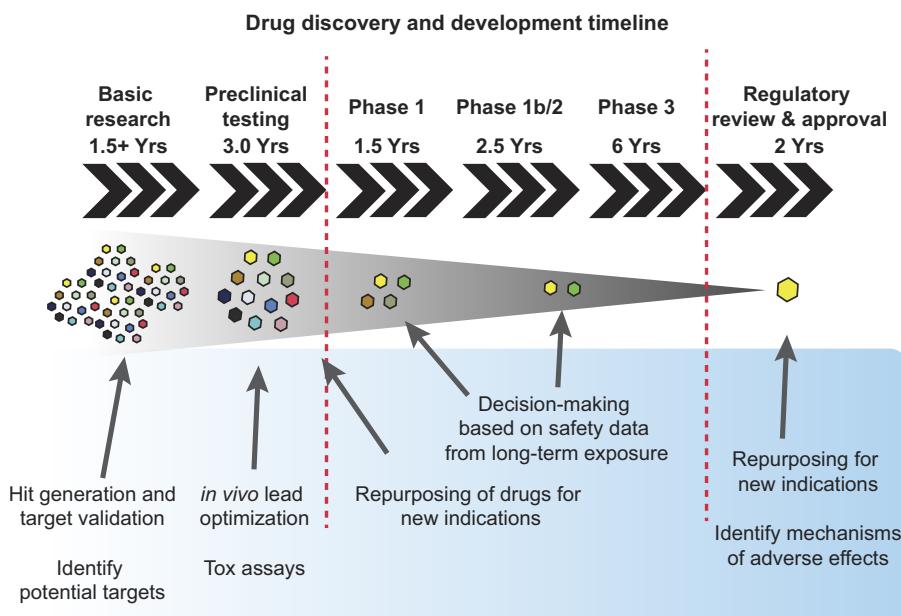


FIGURE 9. Zebrafish assist in all drug discovery and development phases. Fundamental and pre-clinical research using zebrafish generate new targets and help to validate predicted targets *in vivo*. Cornerstones of the traditional R&D pipeline including lead optimization, toxicology testing, and long-term efficacy as well as safety studies are being developed in preclinical zebrafish assays. Drug repurposing can match existing drugs with potentially new clinical indications through screening in disease models. Incorporating whole-organism testing into traditional R&D pipelines early in the process can help critical decision-making and avoid late-stage failure of new chemical entities.

In this section, we will highlight zebrafish strategies that complement traditional R&D pipelines. In some cases, as discussed in the previous section, zebrafish disease models can be used to discover clinically approved drugs that suppress the pathological progress of the disease, and thereby dramatically reduce the time from preclinical to clinical studies (**FIGURES 9 AND 10**). We summarize current challenges including the relatively low throughput compared with *in vitro* screens while highlighting recent progress to leverage robotics and automated image analysis software to narrow down the gap between *in vivo* and *in vitro* throughput capacities.

A. Drug Discovery in Zebrafish Leveraging Organismal Complexity

Along the drug development pipeline, the filtering of hit compounds based on *in vivo* efficacy and toxicity is still considered as one of the most challenging decisions (255). Involving whole-organism biology early on can provide a better understanding of difficult-to-predict variables such as bioavailability, biotransformation, xenobiotic defense mechanisms, as well as homeostatic loops across tissues that abolish or even potentiate the efficacy of a drug, or that may contribute to toxicity or off-target effects. However, early *in vivo* testing of new chemicals is limited by practical, cost, and ethical concerns to only test a handful of hit compounds in mice or rats.

In addition to evaluating efficacy and toxicity, a clear strength of whole-organism screening is the ability to reveal

effects of compounds on cells outside the specific tissue of interest. Whereas cellular screens can only capture cell-autonomous responses to drugs, whole-organism screens can discover molecules that act through a cell type different from the affected cell population via hormonal, nervous, immunological or paracrine mechanisms. This concept is of particular importance for situations where resident cell types have to replace, repair, or remove cells that do not function properly. Examples are immune cells that orchestrate removal of cells while stimulating niche-resident stem cells to replace tissue in response to injury (25, 323).

Most zebrafish screens are carried out during the first week after fertilization, while the animals are still small, transparent, and do not require feeding (319). Several phenotypic readouts and screening strategies exist: wild-type embryos can be used to perform behavior-related readouts (189), anatomic readouts, physiological readouts such as heart rate or blood flow (219, 302), or readouts that can be visualized by simple staining like *in situ* hybridization (276). However, most screens use readouts that are based on transgenic fluorescent or bioluminescence reporter systems. Using bioluminescence allows a relatively quick assessment of promoter activity using conventional plate reader instruments, but requires a functional promoter of interest (13, 124). Fluorescence-based readouts allow high-content visually derived information, but are often of lower throughput, need longer lead-time for transgenic generation, and require more optimization of image acquisition and data handling. Once a model is up and running, it can be used to screen many different chemical libraries (see **TABLE 9**).

**Zebrafish model of human disease
biosensor line of a pathological process**

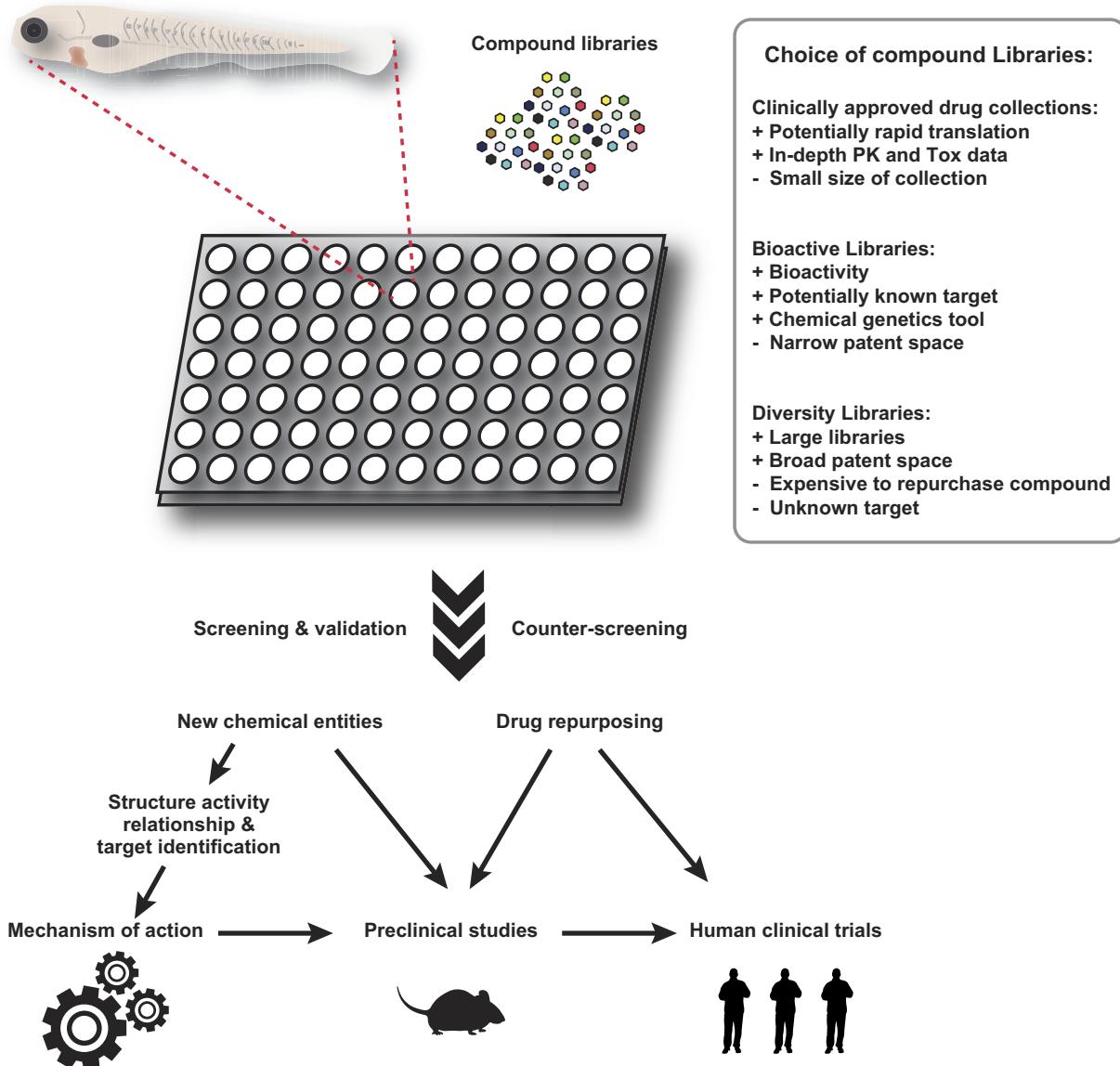


FIGURE 10. From human disease to therapies using zebrafish. In many cases, disease models can be used to develop screening paradigms for suppressor molecules of pathological hallmarks. This emerging concept has been shown to facilitate the transition from preclinical to clinical trials, in particular in areas of unmet clinical need through screening of clinically approved drug collections. Advantages and disadvantages of available chemical libraries are summarized in the box.

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One of the first screens on a zebrafish disease model showed that out of 5,000 screened compounds, two molecules were able to suppress aortic coarctation, a rare pediatric malformation, and to restore blood circulation in *gridlock/hey2* mutants (302). With time many other screens followed, and the sophistication of disease models as well as the screening readouts evolved at a fast pace (TABLE 8). For example, Asimaki et al. (13) modeled arrhythmogenic cardiomyopathy by overexpressing the pathogenic 2057del2 plakoglobin variant specifically in cardiac muscle. These transgenic zebrafish show abnormal cardiac physiology by 48 hpf and develop profound cardiac hypertrophy and arrhyth-

mia by 5 wk of age, mirroring organ-wide human disease phenotypes in a way that an *in vitro* or cell culture models would not. In an elegant screen a luciferase reporter line for natriuretic peptide b *Tg(nppb:Luc)* was used as a surrogate to monitor cardiomyocyte dysfunction at a scale that allowed the authors to carry out a high-throughput screen. Subsequently, a small molecule, SB216763, was identified that reversed arrhythmic contractions, likely through modulating cellular trafficking of components of intercalated disks and thereby restoring cell-cell connectivity while sparing the organism of obvious off-target toxicity (13).

Table 7. Computational survey of cardiometabolic diseases modeled in zebrafish

Disease Class	Disease Examples	Number of Citations	Number of Genes	First Citation Year	Representative Genes
Cardiomyopathy/heart Failure	Dilated, hypertrophic, restrictive, amyloid, noncompaction, arrhythmogenic, toxic, muscular dystrophy-associated	121	105	1999	<i>ERBB2, EYA4, HAND1, JUP, MYH7, TCAP, TNNT2, TTN</i>
Arrhythmia	Long QT syndrome, short QT syndrome, Brugada syndrome, sick sinus syndrome, atrial fibrillation, other conduction disease	138	91	2000	<i>GREM2, KCNH2, KCNK1, KCNQ1, MYL4, PITX2, SCN5A, TBX5</i>
Myocardial injury and regeneration	Myocardial injury, cardiac regeneration	144	62	2002	<i>ALDH1, CAV1, ERBB2, GATA4, IGF1R, NFKB, NRG1, WT1</i>
Structural and congenital heart disease	Ciliopathies, heterotaxy, RASopathies, cohesinopathies, mitral valve prolapse, endocardial cushion defects, septal defects, diGeorge syndrome, tetralogy of Fallot	74	97	1999	<i>A2ML1, BBS, CEP290, DCHS1, DHAND, MEF2C, NIPBL, NPHP4, PTPN11, RAD21, RAF1, RIT1, TBX1</i>
Rare congenital syndromes	CHARGE syndrome, congenital diaphragmatic hernia, Holt-Oram syndrome, hypoplastic left heart syndrome, orphan congenital syndromes	114	157	1999	<i>BMP4, CHD7, FOXH1, GATA4, SAL4, SOX10, TBX1, TBX5, TBX20</i>
Vascular disease	Cerebrovascular disease, atherosclerosis, fibromuscular dysplasia, aortic and branchial arch abnormalities, pulmonary hypertension, hereditary hemorrhagic telangiectasia, other vascular disease	76	118	1996	<i>BARX1/2, CXCR4, DLL4, EDNRA, EPHB4, FGF8, FLT4, GATA1, GATA2, HAND2, NOTCH, RNF213, SOX18, VEGF</i>
Diabetes, obesity, insulin resistance	Diabetes, obesity, insulin resistance	247	300	1996	<i>AHCY, BBS, FABP, FTO, GCG, INS, LEP, MTOR, PCK1, PDX1, POMC, PPARG, PTF1A, SLC16A6</i>
Lipid metabolism	Hepatic steatosis, hyperlipidemia, hypertriglyceridemia	167	239	1974	<i>APOB, APOE, ATF6, CETP, FASN, HMGCR, INS, LDLR, SCAP</i>
Inborn errors of metabolism	Enzyme deficiencies, mitochondrial deficiencies, hemochromatosis, Barth syndrome, hereditary spastic paraparesis	121	176	2000	<i>ALAS2, ETFA/B, FKRP, GLRX5, HFE, KIF, SPAST, TAZ</i>

A custom Python script was written to search MEDLINE and return number of citations, PMIDs, and genes listed in the titles or abstracts as search results. Searches were specifically designed for zebrafish models of human disease. PMIDs returned by the Python script were entered into <https://www.ncbi.nlm.nih.gov/pubmed/> and the corresponding abstracts were validated for relevance.

B. Matching Old Drugs With New Diseases

Drug repurposing (also known as drug repositioning) refers to the identification of a novel indication for a drug that has previously been approved for human use by regulatory authorities. Repurposing can dramatically reduce drug development costs because the toxicology and safety profiles are

already well defined. Zebrafish disease models screened with preapproved drug libraries for their ability to suppress a given disease phenotype are one step closer to clinical therapy. For example, the screens using *kcnh6* mutants to find suppressors of the long QT phenotype mentioned above showed that flurandrenolide and dexamethasone, glucocorticoids for which there is vast clin-

Table 8. Representative chemical screens to identify disease modifier compounds

Read-out	Description	Key Findings Reported	Reference Nos.
Cardiovascular			
Suppression of an aortic coarctation phenotype in <i>hey2</i> mutants	5,000 small molecules screened for compounds that restored blood circulation in <i>hey2</i> mutants.	The first zebrafish screen to report the suppression of a disease phenotype <i>in vivo</i> through organismal phenotypic screening.	302
Rescue of doxorubicin-induced cardiotoxicity	Suppression of cardiac function defects induced by doxorubicin. 3,000 small molecules screened.	Identification of Visnagin, a small molecule suppressor of cardiotoxic effects of doxorubicin that does not interfere with its chemotherapeutic effects.	219
Restoration of rhythmic heart contractions	Screen for compounds that restored coordinated heart beats in mutants that display Ca^{2+} extrusion defects.	A small molecule, efsevin, potentiates VDAC2-mediated Ca^{2+} uptake into mitochondria.	357
Restoration of normal cardiac rhythmicity in <i>kcnh6</i> mutants, a model of Long QT Syndrome Type 2	1,200 molecules screened visually for compounds that suppress a 2:1 atrioventricular block.	Flurandrenolide and 2-MMP shorten the ventricular action potential and suppress the Long QT syndrome phenotype.	298
Bioluminescence readout of a cardiac stress-signal in a disease model for arrhythmogenic cardiomyopathy	4,200 molecules screened for suppression of a <i>nppb</i> :luc signal in zebrafish overexpressing mutant 2057del2 plakoglobin.	A small molecule, SB216763, prevented heart failure and reduced mortality.	13
Suppression of hyaloid vessel angiogenesis in <i>Tg(fli1:EGFP)</i> zebrafish	Angiogenesis inhibitor screen carried out in zebrafish; hits were further tested for suppression of angiogenesis in mammalian cells and a mouse model of retinopathy.	Identification of Quininib, a small molecule inhibitor of the cysteinyl leukotriene receptors 1 and 2, that inhibits revascularization in a model of oxygen-induced retinopathy in mice.	320
Metabolism			
Enzymatic measurements of larval glucose levels	Screen of 13,120 compounds for modulators of whole body glucose levels.	Identification of PTPMT1 as target of a novel glucose-lowering molecule, alexidine.	268
Bioluminescence-based read-out of hepatic gluconeogenesis	A <i>pck1</i> :luc2 reporter line used to screen 2,460 molecules for modulators of a gluconeogenic fasting response.	Translocator protein (TSPO) ligands induce fasting metabolism in zebrafish and mice.	124
Intestinal lipid absorption	A fatty acid labeled fluorophore dye was used to monitor intestinal lipid uptake from the medium into the gallbladder; 3840 small molecules screened.	Identification of known and several previously unknown small molecules that inhibit intestinal lipid absorption.	58
Regeneration of beta cells using a genetic cell-ablation system and fluorescently-labeled beta cells	Screen of 7,000 compounds for enhancers of beta cell regeneration.	Adenosine signaling promotes beta cell regeneration in zebrafish and mice.	5
Differentiation of beta cell progenitors	Screen of 3,131 compounds for induction of secondary islet formation from intrapancreatic duct progenitors without affecting global Notch signaling.	Identification of two activators of beta cell differentiation, MPA and DSF.	327
Beta cell numbers using a fluorescent reporter	3,348 compounds screened at 6 concentrations in a rapid semi-automated process.	Development of a quantitative "high-throughput" screen in zebrafish with up to 200,000 organisms quantified in one day.	412

cal experience, efficiently shorten ventricular action potentials (226, 298).

Several libraries are available that contain clinically approved compounds, including those of the National Institutes of Health that are available to academic researchers at cost (**TABLE 9**). However, it is an oversimplified assumption that drug repurposing automatically facilitates clinical trials, because the clinically accepted safety and toxicology profile of a drug depends largely on its disease indication. In other words, a compound that extends survival and quality of life of a terminal cancer patient may be indicated in this case, but severe adverse effects may exclude it from application in a generally healthy patient population for a less severe disease. Nevertheless, drug repurposing is an interesting area in which zebrafish are likely to take center stage among patient-derived genomics data, disease modeling, and drug discovery (67, 226, 442).

C. Target Deconvolution

The path to identifying the target of a compound found in a phenotypic screen can be uncertain and time consuming. The choice of compound library therefore has an important impact on the likelihood of finding a drug-target match, which is particularly true for *in vivo* phenotypic screens that in most cases are low-to-medium throughput. For example, compound libraries that are rich in information on the chemical structures are most likely to have known targets (50). These “bioactive” libraries contain clinically approved drugs, drugs that have entered clinical trials but have failed for lack of efficacy or toxicity, and experimental molecules that have been discovered as relatively specific for a target of interest. On the other hand, screening libraries of unknown compounds increase the chance to find true novelty and also increase the chance to operate within an open patent landscape, if desired. Advantages of different compound libraries are summarized in **FIGURE 10** and **TABLE 9**.

Several complementary strategies exist to identify the target of an unknown molecule. For example, *in silico* predictions can match chemical structures to protein pockets, or match the structure of an unknown molecule to that of a similar one that has a well-defined target; both approaches have been used successfully in the context of whole-organism screens (195, 206, 422). Some molecules can be synthesized and conjugated to a substrate for subsequent affinity-chromatography pull-down and identification of the target, without affecting the molecule’s native biological activity (127, 268). Lastly, compounds can be screened against large panels of assays that probe modulation of protein superfamilies, including kinases, phosphatases, and G protein-coupled receptors (GPCRs) to identify the target and its relative selectivity over related targets. For example, Williams et al. (425) identified a new molecule, eggmanone, which phenotypically echoed genetic deletions of the hedge-

hog pathway. By screening the lead compounds against a large panel of phosphodiesterases, the authors found eggmanone to selectively inhibit three distinct phosphodiesterases, including PDE4D3 with an IC₅₀ of 72 nM. Counter-screening against a panel of 442 kinases, 158 GPCRs, and 21 phosphatases confirmed selectivity of the compounds to PDE4D3. Once a plausible target is identified, genome-editing approaches can be used in cell lines or in zebrafish to validate the dependence of the phenotypic readout on the target.

Another emerging concept to match a molecule of unknown structure to a target is “bar coding” to show that the unknown molecule performs similarly to modulating a matrix of readouts as a well-defined molecule (38, 189, 190, 239). Bar coding is especially helpful when the efficacy of a particularly potent molecule may in fact not be mediated by one target, but several synergistic targets—the basis of polypharmacology (38, 126, 239). Whole-organism screening is also well suited for a polypharmacology approach because the synergistically acting targets may function in different tissues. Of course, the possibility of undesired adverse effects increases with the number of bioactive targets, i.e., the “dirtier” a drug is, the more important it is to assess toxicological effects in whole-organism models early during its development.

D. Efficacy and Route of Administration

Zebrafish screens are commonly carried out at one concentration (in most cases at 10 μM), with the exception of a few semi-automated screens that were able to screen a titration range (412). The compounds of the stock plates are commonly dissolved in DMSO and reach final DMSO concentrations of 0.1–2% in the screening plate. The compound libraries contain chemicals with different physicochemical characteristics ranging from hydrophilic to relatively hydrophobic. As a result, it is difficult to predict whether a particular chemical enters into the zebrafish orally, topically, or through the gills. In addition, it is not possible to predict the concentrations within zebrafish tissues, in particular because measuring drug concentrations in whole larvae is often misleading as the chemicals frequently attach to the skin. To bypass this latter problem, a group developed a microneedle biopsy in the yolk sac followed by mass spectroscopy to determine whether the compound has truly been absorbed into the zebrafish (288). Another means to test whether a compound is orally available is to administer it to adult zebrafish via gavage or gelatin-based food delivery. Oral gavage of adult zebrafish can be performed on zebrafish that are anesthetized and mounted in an incised sponge (61, 71). An alternative, although less precise, is the preparation of a mix of low-melting gelatin mass (such as Gelly Belly, Florida Aqua Farms) and the compound of interest. The solidified gelatin can be cut into small pieces and fed to adult zebrafish (348). Both techniques greatly

Table 9. Selection of available chemical libraries

Type	Name	Provider	Size	Description
Bioactives	TocrisMini	Tocris	1,180	Drugs, screening hits, and experimental small molecules with known bioactivity. Off-the-shelf repurchasing of large quantities.
Bioactives	SigmaLopac	Sigma	1,280	Drugs, screening hits, and experimental small molecules with known bioactivity. Off-the-shelf repurchasing of large quantities.
Bioactives	Prestwick Chemical Library	Prestwick	1,280	Approved drugs by FDA, EMA, and other agencies. Off-the-shelf repurchasing of large quantities.
Bioactives	Bioactive Compound Library	SelleckChem	2,100	Some FDA approved drugs, natural products, and other molecules with known bioactivity. Off-the-shelf repurchasing of large quantities.
FDA-approved drugs	FDA-approved Drug Library	SelleckChem	978	FDA-approved drugs. Off-the-shelf repurchasing of large quantities.
Targeted library	Screen-Well Compound Libraries	Enzo	>2,500 total	Selection of libraries containing bioactive lipids, Nuclear Receptor Ligands, GPCR ligands, among others.
Targeted library	Screening Libraries	Cayman Chemicals	NA	Selection of libraries containing epigenetics, kinase and stem cell modulators, bioactive lipids, prostaglandins
Plant crude extracts		TimTec	NA	Fractions of plant extracts. Difficult to identify active ingredient(s); potential reproducibility issues.
Pure natural products	Natural Product library	TimTec	800	Single compounds derived from the natural space; potentially expensive to purchase larger quantities or even impossible to extract sufficient amounts for preclinical studies.
Pure natural products	Natpure	Charles Rivers	14,100	Single compounds derived from the natural space; potentially expensive to purchase larger quantities or even impossible to extract sufficient amounts for preclinical studies.
Pure natural products	Natural Compound Library	Chromadex	1,850	Single compounds derived from the natural space; potentially expensive to purchase larger quantities or even impossible to extract sufficient amounts for preclinical studies.
Plant crude extracts	Botanical Extract Libraries	Chromadex	>1,600	Fractions of plant extracts. Difficult to identify active ingredient(s); potential reproducibility issues.
Pure natural products	Screen-Well Natural Product library	Enzo	502	Single compounds derived from the natural space; potentially expensive to purchase larger quantities or even impossible to extract sufficient.
Plant crude extracts	Greenpharma Extract Library GPEL	GreenPharma	NA	Fractions of plant extracts. Difficult to identify active ingredient(s); potential reproducibility issues.
Human Endogenous Metabolites	Human Endogenous Ligand Library GPLL	GreenPharma	400	Metabolite-like human endogenous ligands.
Diversity	NExT diversity libraries	National Institute of Health	80,000	Large libraries with potential for novelty; unknown bioactivity; expensive to repurchase or synthesize compounds.
Diversity	AXX ^{DIV2.0}	AXXAM	12,000, 48,000, or 180,000	Large libraries with potential for novelty; unknown bioactivity; expensive to repurchase or synthesize compounds.

Continued

Table 9.—Continued

Type	Name	Provider	Size	Description
Diversity	Express-Pick Collection, Core Library Stock	Chembridge	Custom; >1,000,000	Large libraries with potential for novelty; unknown bioactivity; expensive to repurchase or synthesize compounds.
Diversity		Exqiron	Custom; >250,000	Large libraries with potential for novelty; unknown bioactivity; expensive to repurchase or synthesize compounds.
Diversity	TimTec	TimTec	Custom; >>600,000	Large libraries with potential for novelty; unknown bioactivity; expensive to repurchase or synthesize compounds.

expand the use of zebrafish for preclinical studies as they allow long-term drug administration.

E. Toxicology

Several important studies have been carried out to date that illustrate how zebrafish can be leveraged to detect the adverse toxic effects of some small molecules (63, 115, 222, 275). A seminal study found that thalidomide exerts teratogenic effects during zebrafish development that resemble those found in human newborns whose mothers were treated with the drug (under the brand name Contergan) (165). Thalidomide was widely prescribed in Europe in the 1950s and 1960s as a sedative or anti-emetic during pregnancy. More than 10,000 children were born with severe upper and lower limb malformations because of potent teratogenic effects that occurred in humans, but were not observed in mice or rats (294). Interestingly, zebrafish treated with low levels of thalidomide exhibited dose-dependent growth defects during limb formation.

A thalidomide-binding protein, Cereblon (CRBN), was identified, and *crbn* loss-of-function in zebrafish mimicked the limb outgrowth defects seen with thalidomide treatment (165). Importantly, the limb defects in thalidomide-treated zebrafish embryos could be rescued by injecting mRNA encoding a mutated protein unable to bind thalidomide. In contrast, wild-type mRNA was unable to rescue the toxic effects, suggesting that CRBN is the direct target of thalidomide teratogenicity. These results are significant because they show that drug treatments in zebrafish can reveal teratogenic effects that may be overlooked when focusing teratogenicity studies exclusively on rodent models. In addition, the genetic tractability of zebrafish greatly facilitates the distinction of therapeutically relevant targets from secondary targets that mediate toxicity, raising the possibility to use this model to develop new generation drugs that are specific for the primary target.

Clinically, the benefits of some drugs outweigh their toxic effects, and these drugs remain an important therapeutic choice. One such example is doxorubicin, a chemothera-

peutic of the anthracycline class that is used to treat malignancies, including breast cancer and lymphomas, but causes cardiomyopathy as an important adverse effect. Recently, zebrafish were leveraged to identify a potential adjuvant treatment that reverts doxorubicin-induced cardiomyopathy (219). First, the authors established a screening paradigm in which doxorubicin-induced cardiotoxicity could be scored by visually assessing contractility and blood circulation of live zebrafish larvae. Among 3,000 screened compounds, 8 were identified as chemicals that efficiently protected from cardiac dysfunction. The authors further evaluated two of the hit compounds, visnagin and diphenylurea, which showed highly potent cardioprotective effects. Importantly, these compounds did not interfere with the chemotherapeutic effects of doxorubicin in zebrafish and mouse xenotransplantation models of leukemia and breast cancer, respectively.

Whereas these and other examples constitute a proof of concept that some clinically relevant toxic or teratogenic effects can be similar between zebrafish and humans, it is less clear how widely zebrafish can be used for unbiased toxicity screening of new chemical entities. Any drug discovery pipeline would benefit tremendously from the ability to reliably predict toxic effects. Such a comprehensive forward toxicity profiling remains a major challenge for the future, and the zebrafish model promises to be part of the solution (111, 226).

F. Scaling-Up the Throughput

Although the exact definition of high-throughput screening (HTS) is not carved in stone, 10,000–100,000 compound tests per day is commonly considered to be at HTS scale. Common zebrafish screens average around 2,000–5,000 compounds in total, and in most cases at a single concentration. The largest screen published to date tested 30,000 small molecules over several months to identify compounds that interfere with signaling pathways critical for embryonic development (425).

A recent screen studying the formation of secondary islets in the pancreas was able to improve the automation of zebrafish handling, drug administration, and the quantification of the reporter to scale up the throughput to test ~3,000 compounds in 16 replicates and at 6 different concentrations, cumulating to 500,000 zebrafish exposed to chemicals (412). The titration of dosage allowed determination of dose-response curves and width of the therapeutic window, thereby adding valuable information to preselect the best hits before validating the compounds in counter screens. Despite this progress, the overall throughput is largely limited by several factors, including the number of embryos that can be produced, the selection of healthy individuals from a pool of wild-type or fluorescently labeled reporter zebrafish, the loading into microtiter plates, and ultimately the complexity of the readout.

In a different approach, a system was developed to inject *Tuberculosis marinum* bacteria into the yolk of developing embryos, followed by an automated “health check,” distribution of embryos into 96-well plates, and automated analysis of fluorescence intensity of the bacterial content (42). This approach can be combined with chemical screening to identify new regulators of bacterial replication within a host organism. Instead of microorganisms, the same setup can be used to inject morpholinos to knock down genes with sufficient throughput to screen on a transient loss-of-function model.

Other efforts are geared towards automating image analysis in zebrafish by addressing the challenge of orienting the larvae and visualizing fluorescently labeled structures in three dimensions (293, 309). An instrument named the VAST BioImager mounts on a confocal or fluorescent microscope, loads a zebrafish larva in a capillary tube, and moves it along the tube. The setup can be programmed to apply pattern-recognition software for automated, user-defined positioning and image capture.

So far, automated efforts have been focused on probing preselected chemical collections with mostly known bioactive effects and a high proportion of clinically approved drugs. The goals are of chemical genetic interest, for example, to dissect a signaling pathway or discover a lead compound (90). The restriction of the chemical collection to bioactive targets and the mostly academic perspective on what is considered a hit compound limits to some extent the necessity for HTS. Nevertheless, drug discovery approaches in zebrafish will benefit from an increasing level of sophistication, and efforts are underway to increase the throughput of compounds tested and data points that can be analyzed.

V. SUMMARY AND CONCLUSIONS

Zebrafish have developed at a breathtaking pace towards being a principal animal model in biomedical research.

Studies of several zebrafish disease models have shown the high relevance of this approach, and some of the first drugs discovered in zebrafish are now in clinical trials further underscoring the translational impact of this model. Reverse genetic techniques have recently been added to the suite of genetic manipulations that can be performed easily, space- and cost-efficiently in zebrafish, opening up indefinite possibilities to design preclinical disease models. Many practical and technical considerations go into generating a new disease model, as summarized in FIGURE 4. Physiologists and clinicians within the zebrafish community can help with genetic design and rigorous phenotyping of an appropriate model. Generating gene knockout models for newly identified disease-associated SNPs will remain a cornerstone of zebrafish research in the years to come.

Several questions remain for the future. How much clinical relevance can we extract from studying a relatively simple, mostly monogenic, disease modeled in zebrafish? Certainly, finding solutions for complex diseases whose progression is defined by the confluence of environment, behavioral, nutritional, genetic, and epigenetic factors is extremely challenging. However, on the clinical side of biomedical research, a trend has emerged towards improving the resolution of human disease phenotyping, subcategorizing disease, and reducing population complexity to better translate knowledge from preclinical models to humans. This trend is known as personalized medicine, and the power of zebrafish genetics in conjunction with pharmacology will likely help better define personalized medicine approaches and the design of proof-of-concept studies in subpopulations of a disease.

Conducting preclinical research with a reliable impact on human disease is a scientific challenge. With this challenge comes the responsibility to not over-sell or mislead as patients anxiously await for breakthroughs that promise to improve the quality of life. Failures in translational research undermine trust and ultimately consent to perform trials. Thus, with the genetic and phenotyping capabilities currently available to the zebrafish community, rigorous research design is a must. Zebrafish are still frequently used to complement human genetics studies with morpholino experiments to “confirm” a biased hypothesis through assays that at best distantly resemble human biology, like yolk sac consumption as a readout for adipose tissue plasticity or pericardial edema for any kind of heart condition. The zebrafish community has the potential to meet the challenge for rigorous preclinical research, given its ability to generate hundreds or even thousands of gene knockouts and to systematically profile their phenotypes to relate them to disease conditions.

To date, the capacity for in-depth phenotyping when it comes to physiology and tissue-specific biochemistry still lags behind the level of resolution in rodents. However,

rather than competing with preclinical rodent models, zebrafish provide a powerful complementary platform. With increasing depth of phenotyping paved by nanotechnology solutions and sophistication of high-throughput and high-content screening efforts, we expect the zebrafish to further improve its position as a prime preclinical animal model, and to benefit human health.

ACKNOWLEDGMENTS

We apologize to the authors whose papers we could not reference due to space limitations.

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GRANTS

We thank the Max Planck Society for financial support. R. Arnaout was supported by National Heart, Lung, and Blood Institute Grant K08HL125945 and American Heart Association Grant 15GPSPG238300004.

DISCLOSURES

P. Gut is an employee of Nestlé Institute of Health Sciences, part of Nestlé Group. S. Reischauer, R. Arnaout, and D. Y. R. Stainier have no conflicts of interest, financial or otherwise.

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