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Developing zebrafish disease models for *in vivo* small molecule screens

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The zebrafish is a model organism that allows *in vivo* studies to be performed at a scale usually restricted to *in vitro* studies. As such, the zebrafish is well suited to *in vivo* screens, in which thousands of small molecules are tested for their ability to modify disease phenotypes in zebrafish disease models. Numerous approaches have been developed for modeling human diseases in zebrafish, including mutagenesis, transgenesis, pharmacological approaches, wounding, and exposure to infectious or cancerous agents. We review the various strategies for modeling human diseases in zebrafish and discuss important considerations when developing zebrafish models for use in *in vivo* small molecule screens.

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Introduction

The zebrafish (*Danio rerio*) has proven to be a powerful vertebrate model for disease modeling and chemical screening. The advantages of the zebrafish model include the high degree of conservation between zebrafish and human genomes and molecular signaling networks, its small size, external fertilization, rapid development, optical transparency at early developmental stages, high reproduction rate, and low maintenance cost. Together, these attributes enable large-scale *in vivo* chemical screening via mass production of zebrafish, systematic exposure to small molecules in multi-well plates, and rapid phenotypic assessment. Dozens of successful *in vivo* screens ranging from hundreds to tens-of-thousands of small molecules have been reported [1,2]. It is worth noting that some small molecules identified through zebrafish screens have been shown to be effective across different animal species and have even led to human clinical trials (Table 1).

One particularly useful approach is to model a human disease in zebrafish, then screen small molecule libraries for compounds that suppress the disease phenotype. This review aims to provide an overview of the different approaches taken in generating zebrafish disease models for the purpose of phenotypic chemical screening (Figure 1). By no means comprehensive, we will discuss only a few examples to illustrate the various approaches. The advantages, limitations and suitability of each approach will also be discussed.

Genetic models

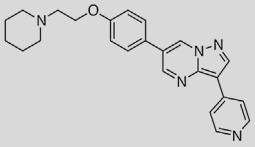
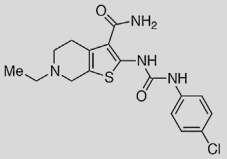
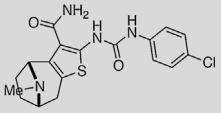
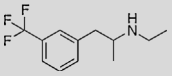
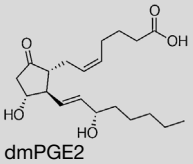
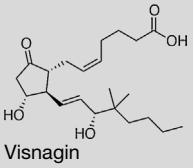
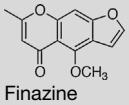
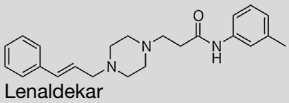
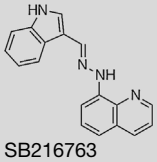
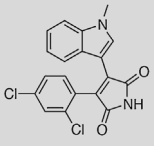
Perhaps the most obvious approach for creating screenable disease models in zebrafish is a genetic approach. Approximately 82% of human disease-related genes (listed in OMIM) have a zebrafish ortholog [3•], and thousands of zebrafish gene mutations have already been generated and cataloged. Identifying small molecule suppressors for the hundreds of known human genetic disorders is a daunting task, but the efficiencies of effort and cost provided by the zebrafish make it possible to imagine such an audacious undertaking.

The earliest zebrafish genetic mutant used for a chemical screen was the *gridlock* mutant, which harbors a mutation in the *hey2* gene. *Gridlock* mutant embryos produce insufficient arterial progenitor cells, and as a result develop an aortic constriction resembling human coarctation of the aorta [4]. Independent small molecule screens identified compounds that completely and permanently rescued the aortic defect by stimulating arteriogenesis [5,6•], an effect that was conserved in mice [7]. Since then, several other zebrafish genetic models have been used for chemical screening, including models of Long QT syndrome, cardiac arrhythmia, Duchenne muscular dystrophy, and Dravet syndrome [8•,9–11].

The gridlock suppressor screen took advantage of the fact that homozygous gridlock mutants are viable and fertile, allowing homozygous breeding pairs to be established. Such is not always the case; many mutants are homozygous lethal, so new homozygotes can only be produced by incrossing of heterozygotes. This phenomenon represents a major practical limitation for zebrafish screening because only 25% of a heterozygous incross are homozygous mutants, making it difficult to distinguish between a homozygous mutant that has been 'rescued' by a small

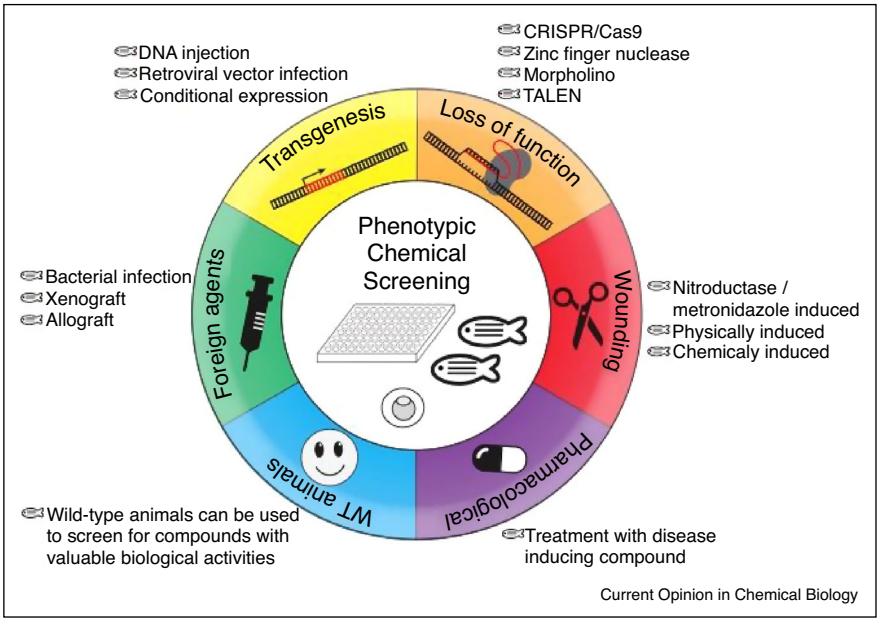
Table 1

Selected small molecules discovered from zebrafish small molecule screens

Compound	Biological activity and status	Reference
Dorsomorphin 	Dorsomorphin and its derivatives are inhibitors of Bone Morphogenetic Protein (BMP) receptors. These compounds were selected by US National Institutes of Health for preclinical development for fibrodysplasia ossificans progressiva (FOP) and anemia of inflammation.	[74•]
ORC-0001 (PROTO-1) 	ORC-0001 and its derivative ORC-13661 were discovered to be protective against aminoglycoside antibiotic (AGA)-induced hair cell death. A clinical trial of ORC-13661 is underway.	[79,80]
ORC-13661 		
Fenfluramine 	Fenfluramine, a serotonin reuptake inhibitor, demonstrated efficacy against spontaneous seizures in *scn1lab*^{s552} mutants. It is currently in phase III clinical trials for Dravet Syndrome.	[8•]
Prostaglandin E2 (PGE2) 	PGE2 was found to induce stem-cell production. The long-acting derivative of PGE2, 16,16-dimethyl-PGE2 (dmPGE2), has now advanced to a phase II clinical trial as a therapy for patients undergoing umbilical cord blood transplantation.	[73•]
dmPGE2 		
Visnagin 	Identified to protect against doxorubicin-induced cardiomyopathy in zebrafish and mouse models.	[25]
Finazine 	Finazine was found in a screen of zebrafish behaviors to elicit a behavioral profile similar to other antipsychotics, suggesting it might also have antipsychotic activity, a hypothesis confirmed in a mouse model.	[81]
Lenaldekar 	Lenaldekar (LDK) was discovered to selectively eliminate immature T cells in the developing zebrafish and as a potential treatment for leukemia.	[82]
SB216763 	SB216763 is a glycogen synthase kinase-3 (GSK-3) inhibitor. From a zebrafish screen, SB216763 was found to improve features of arrhythmogenic cardiomyopathy.	[21•]

molecule and its unaffected heterozygous or wild-type sibling. Various approaches can be used to overcome this lethality problem. Stern *et al.* screened the progeny of a heterozygous incross of *crash-and-burn* mutants, but rather than screening just a few animals per compound as is common in zebrafish screens, they screened each compound against a pool of ~20 zebrafish so that the probability of any pool containing at least one homozygous

Figure 1



Schematic representation of the six different general approaches for generating zebrafish disease model for chemical screening.

embryo was high [12]. Although effective, this approach necessitated the screening of many more animals per compound than is typically necessary when all animals are homozygous. Other groups have overcome the lethality issue by transplanting mutant germ cells into wild-type animals to create fertile, viable breeding pairs that produce uniformly homozygous mutant offspring [13]. Similarly, Mullapudi *et al.* transplanted wild-type endoderm into non-viable *insulin* mutants to enable them to survive to sexual maturity and produce large numbers of homozygous offspring for a chemical screen for insulin-independent modulators of glucose homeostasis [14]. Additionally, recent advances in generating conditional genetic alleles in zebrafish will likely enable circumvention of the lethality problem in chemical screening, greatly expanding the number of genetic disorders that can be tackled [15].

Most of the chemical suppressor screens undertaken to date have utilized genetic mutations identified through forward genetic screens and large-scale mutagenesis projects. Importantly, the advent of robust reverse genetics in zebrafish using TALENs and CRISPR-Cas9 dramatically expands the possibilities for disease modeling [16•,17•,18•]. It is now relatively simple to generate null alleles for genes of interest when such alleles are adequate disease models. However, mimicking-specific human disease alleles, including specific point mutations and gain-of-function (GOF) mutations, remains more challenging. Various approaches for precise production of non-INDEL alleles have been developed, but further improvement in efficiency is needed before widespread adoption can occur [19,20]. In addition, most genetic models used to date produce a relatively profound, embryonic phenotype, whereas genetic mutations that produce subtle or adult phenotypes have largely eluded chemical screening. Development of technologies for screening subtle, adult phenotypes that are relevant for many genetic disorders remains an important frontier.

Transgenesis

Transgenesis can be used effectively to model human diseases by creating zebrafish lines that enable regulated expression of a disease-causing gene. Unlike the basic mutagenesis and genome editing approaches described above, which have mostly focused on disrupting endogenous zebrafish genes, transgenesis enables expression of genes with a wide variety of activities including GOF alleles, translocation products, and even human disease genes not present in the zebrafish genome. In one elegant example, Asimaki *et al.* created a transgenic line with inducible, cardiac-specific expression of the 2057del2 mutation of the human plakoglobin gene [21•]. This specific mutation causes Naxos disease in humans; overexpression in zebrafish causes related phenotypes, including natriuretic peptide b overexpression, cardiac contractility defects, cardiomegaly, and eventually death. The authors successfully used the transgenic model in a small molecule screen that identified SB216763, a compound that reversed the Naxos-related phenotypes in

transgenic zebrafish and rat cardiomyocytes expressing 2057del2 [21•]. Several other examples exist. A transgenic line in which the human acute myeloid leukemia (AML) translocation product AML1-ETO is expressed in zebrafish from the heat shock promoter was used to screen for potential AML therapeutics [22]. Similarly, a transgenic zebrafish line expressing the human protein TAU-P301L in its neurons was used to identify compounds that reduced pathologic hyperphosphorylation of TAU *in vivo* [23].

The examples above demonstrate various advantages of the transgenesis approach including the ability to make the disease phenotype conditional using inducible promoters or multi-component systems such as Gal4:UAS, thereby circumventing lethality associated with disease gene expression. In addition, they provide control over the location, timing, and quantity of disease gene expression, enabling titration of phenotype severity. This capability can be extremely helpful in ensuring that the disease phenotype is highly penetrant and observable without being too severe to rescue.

One precaution should be noted when using transgene-based disease models: small molecules that appear to be improving a disease phenotype may simply be reducing expression of the transgene. This phenomenon was observed by Yeh *et al.*, who found that some false positive hits from their screen inhibited the heat shock response in zebrafish, thereby blocking expression of the AML1-ETO transgene [22].

Pharmacological models

In some instances, pharmacological treatments cause robust zebrafish phenotypes that are mechanistically linked to human diseases. For example, the GABA antagonist pentylenetetrazole causes neuronal hyperexcitability that has been shown to model some aspects of epilepsy in rodents and zebrafish such as epilepsy-like electrographic discharges and clonus-like convulsions [24]. The β-adrenergic receptor agonist isoproterenol and the chemotherapeutic agent doxorubicin both cause cardiac dysfunction, cardiomyocyte death, and heart failure in zebrafish, much as they do in rodents and humans, thereby serving as pharmacological models of heart failure [25–27]. Other examples include pharmacological models of biliary atresia, hyperglycemia, and cyanide intoxication [28–32]. Such pharmacological models are generally relatively easy to develop and scale up because they can be performed using wild-type animals. Unfortunately, validated pharmacological inducers of disease phenotypes do not exist for most human conditions. When they do, it should be remembered that small molecule 'hits' that appear to suppress the disease phenotype may be functioning by influencing the metabolism or distribution of the pharmacological agent.

Wounding

Zebrafish have robust regenerative ability [33,34] and leukocyte immune response to tissue injury [35–37]. These attributes make zebrafish wounding (physically or chemically induced) an attractive approach to screen for modulators of regeneration and inflammation. Physically induced wounds are easily achieved with a dissecting microscope and a fine hypodermic needle or scalpel blade [38–42]. For example, to identify compounds that accelerate resolution of inflammation, Robertson *et al.* performed a screen on tail-transected larvae then assessed neutrophil numbers in the injury region [40]. Chemically induced wounding utilizes cell type-specific toxins [43–45]. d'Alencon *et al.* used copper sulfate to selectively damage sensory hair cells then identified immunomodulatory compounds by quantifying the magnitude of the inflammatory reaction [45]. Using a similar approach, Namdaran *et al.* damaged hair cells with neomycin then screened for compounds that modulated their regeneration [44]. Commonly, transgenic zebrafish expressing fluorescent proteins in cells or tissues of interest are used for quantification. Manual wounding offers discrete onset of cellular damage, yet the labor involved often limits the sample size available to screen. Chemically induced cell ablation facilitates larger sample sizes, but damage is often slow to develop and caution needs be taken to identify non-specific cell loss. Cell-specific expression of nitroreductase along with exposure to the prodrug metronidazole is another method to induce cell ablation [46–48]. This approach has already been applied to cell types such as rod photoreceptors [49,50], pancreatic beta cells [51] and hepatocytes [52]. It should be noted that the majority of these wounding approaches are used on early developmental stages when only the innate immune system is functional. The adaptive immune system in zebrafish is not present until 4–6 weeks post fertilization [53]. Furthermore, since zebrafish naturally regenerate most damaged tissue, any inhibitory pathways identified in zebrafish may not translate to agents that promote regeneration in humans.

Exposure to foreign agents

A wide variety of pathogens have been used to generate zebrafish infection models and include bacteria, fungi and viruses [54–57]. Injecting these pathogens directly into the circulation or into different body cavities is most common. High throughput chemical screening can be facilitated using an automated pathogen microinjection system [58•], flow-based fluorescence infection quantification [58•] and bioluminescence-based bacterial load measurements [59]. Alternatively, direct exposure is sufficient for certain pathogens. For example, Dalton *et al.* found that larvae become naturally infected after immersion in media containing *Mycobacterium marinum*, a surrogate of the tuberculosis causing pathogen *Mycobacterium tuberculosis* [59]. If using fish-based infection models, one must consider the lower rearing temperature used for

zebrafish (28°C) when interpreting results and the physiological relevance of the particular immune response under investigation. For example, the zebrafish paralogues of the TLR4 pattern-recognition receptors do not sense LPS [60,61]. Currently, most chemical screening is performed using zebrafish bacterial infection models. Undoubtedly, future work can be extended to include fungal and viral-based models.

Zebrafish-based xenotransplantation models are primarily geared toward developing novel cancer treatments and for personalized medicine [62–65]. Xenotransplantation in zebrafish needs only a small number of cancer cells. This approach offers the advantage of *in vivo* monitoring of cancer cell invasion and metastasis. A variety of zebrafish xenograft models have been established by injecting patient-derived primary cancer cells, patient-derived tumor tissue explants or human cell lines into different sites or into fish at different developmental stages [66–70]. Some of the limitations of this approach include differences between zebrafish and humans with respect to tumor microenvironment [71] and the temperature required for optimal cell growth [72]. With these techniques, the lack of an acquired immune system in early larval stages and immunocompetency issues in the adult must be addressed. The immunocompetency issue can be overcome when clonal syngeneic strains or immune compromised zebrafish are used for allograft cancer cell transplantation. Even though zebrafish cells are used for allogenic transplantations, this approach can interrogate the mechanisms of cancer self-renewal, progression and metastasis [63]. Overall, xenograft and allograft proof of principle show tremendous potential for chemical screening and precision oncology. These models will most certainly aid in the discovery of effective treatments for various cancers in the near future.

Using wild-type animals to screen for disease modifiers

Many diseases arise from the aberrant upregulation or downregulation of signaling pathways important for normal development and function. When phenotypic readouts relevant to the pathway of interest are established, wild-type animals can be used in chemical screens to identify modulators of the disease state. The phenotypic readout could be the over or under production of specific genes or cell types, abnormal development of a phenotype known to be caused by specific signaling pathways, or behaviorally phenocopying a disease state. For example, using *in situ* hybridization to detect changes in expression of mRNAs important for hematopoietic stem cell (HSC) development, North *et al.* discovered that prostaglandin E2 (PGE2) is an efficient inducer of HSC self-renewal [73•]. This discovery has led to the initiation of a clinical trial using PGE2 in umbilical cord blood transplantations. Another example is screening for compounds that cause disruption of dorsoventral axis formation in wild-type larvae. This approach yielded the discovery of a small molecule inhibitor of BMP signaling, dorsomorphin [74•], and an inhibitor of β-catenin function, windorphen [75]. Behavioral responses to different stimuli [76•,77•] or learning behavior [78] can also be used as screening readouts to discover neuroactive drugs and cognitive modulators, respectively. The obvious advantage of using wild-type animals is that no additional time and effort are needed to generate the model. The feasibility of this approach, however, is limited by the availability of observable and quantifiable phenotypic outcomes generated from a wild-type genetic background after compound treatment.

Conclusion

As with all model systems, the zebrafish comes with its own set of advantages and limitations. When selecting or designing a zebrafish disease model for chemical screening, careful considerations should be made regarding the scalability, potential false positive rate, and especially the physiological relevance of the disease model used. The choice of chemical library can also be a critical factor for the success of a screen, but that topic is beyond the scope of this review. Given the numerous established approaches to disease modeling in zebrafish, as reviewed above, and a proliferation of tools for high-throughput embryo handling, robotic injection, and automated image acquisition and analysis, zebrafish-based screening is increasingly becoming a powerful and accessible technology for small molecule discovery.

Conflict of interest statement

Nothing declared.

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