Animal Models of Disease

These systems are becoming increasingly important secondary screens of in vitro hits.

BY CHAOYONG MA

In vitro assays typically rely on simple interactions of chemicals with a drug target, such as receptor binding or enzyme activity inhibition. However, in vitro results often poorly correlate with in vivo results because the complicated physiological environment is absent in the in vitro testing system. Although cell-based assays can provide some information, cultured cells still do not provide physiological conditions and complex interactions among different cell types and tissues. Moreover, cell lines are usually transformed, exhibiting different gene expression and cell cycle profiles than those of cells in the living organism.

PHOTO: PHYLONIX PHARMACEUTICALS

Tanks a lot. An aquaculturist maintains stocks of zebrafish.

There is a growing trend of using human tissues for drug discovery research. Tissues, however, only provide an isolated ex vivo condition, which is not completely representative of in vivo response because drug action often involves metabolism and interplay among different tissues. For example, the effects of a drug on muscle may involve absorption by the intestine and metabolism by the liver. Therefore, results in animal studies are essential to validate HTS (high-throughput screening) hits and exclude compounds with unfavorable ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties, which are responsible for more than half of compound attrition in costly clinical trials.

Currently, in vivo assays are not usually performed until or after the lead optimization stage. This is partly due to the low speed and high cost of conventional animal models (typically rodents) and the relatively high number of preliminary hits from HTS. With alternative small-animal models emerging, however, it is now possible to perform in vivo testing earlier in the process. Thus, researchers have developed model systems using both vertebrates (zebrafish) and invertebrates (the fruit fly, *Drosophila melanogaster*; and the nematode *Caenorhabditis elegans*) for drug screening. The small size, high fecundity, and experimental tractability of these animals enable cost-effective and rapid screening of numerous compounds.

On the fly

The most popular invertebrate model organisms, *Drosophila* and *C. elegans*, have been used extensively in many areas of biological research, especially genetics and development. The use of these models is supported by the existence of highly conserved molecular pathways between invertebrates and humans, such as the MAP kinase pathway (*1*). Combined with the powerful genetics, cellular, and molecular biology tools available, these model systems are very suitable for drug discovery research (*2*, *3*).

Using the fly, researchers have developed models for many complicated pathologies. For example, Raymond Pagliarini and Tian Xu of the Yale University School of Medicine used genetics techniques to explore the complex, multistep processes of oncogenic transformation and metastasis in *Drosophila* (*4*). Similarly, researchers have generated transgenic *Drosophila* lines that overexpress mutated proteins, which causes flies to undergo neurodegeneration and allows them to serve as models for disorders such as Alzheimer's disease and Huntington's disease (*5*). Compared with transgenic mice, transgenic *Drosophila* are much easier to construct.

One *Drosophila* organ that researchers extensively analyze is the compound eye, which develops from a monolayer precursor tissue in the larva and consists of about 800 unit eyes (ommatidia) arranged in a highly accurate pattern (Figure 1). The large number and stereotypic pattern of the unit eyes make it easy for researchers to use the eye phenotype to identify genes interacting with a disease gene or drugs affecting components of the pathway (*6*, *7*).

Several biotechnology companies have developed *Drosophila*-based drug discovery technology platforms. Exelixis has a gene-knockout array generated from a large collection of stocks that each contain a single transposon insertion in the fly genome. This allows researchers to quickly characterize a gene's function using a reverse genetics approach. EnVivo Pharmaceuticals has devel-

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oped models for neurodegenerative diseases based on the expression of human disease genes in the fly. These models mimic the characteristic neuropathology and specific symptoms of the diseases, and they can be used for drug screening. The Genetics Company has developed a high-throughput in vivo system that can be used to screen small-molecule compounds in flies to identify drug leads with better ADME properties.

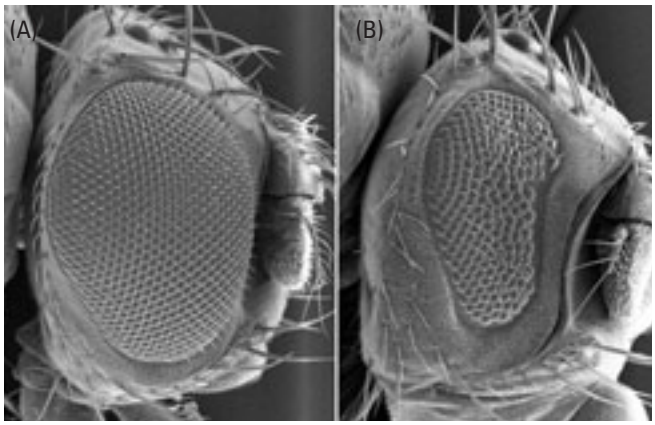


Figure 1. An eye on flies. Researchers use scanning electron microscope imaging to view differences between wild-type *D. melanogaster* (A), which has a normal compound eye with accurate arrangement of unit eyes (ommatidia), and a mutant fly (B), which has a small and rough compound eye with dramatically fewer ommatidia. (Photos courtesy of Kevin Moses, Emory University.)

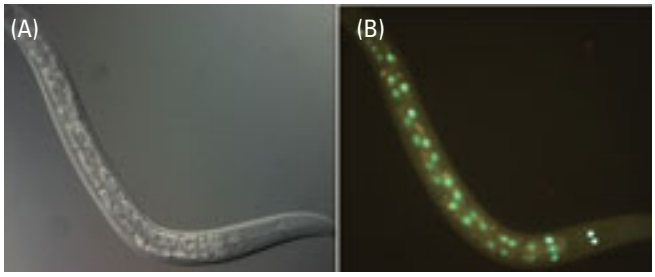


Figure 2. A worm with a view. Researchers use bright-field imaging (A) to see individual cells in *C. elegans* or fluorescent imaging (B) to see particular cells expressing green fluorescent protein. (Photos courtesy of Jae Hyung An and Keith Blackwell, Joslin Diabetes Center, Harvard Medical School.)

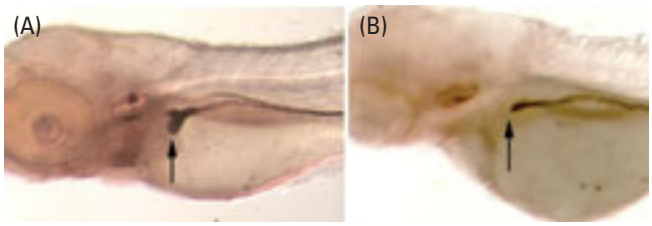


Figure 3. Crystal clear analysis. The see-through nature of the zebrafish allows researchers to distinguish a normal specimen (A) stained with an antibody that highlights the kidney (arrow) from a drug-treated individual (B) with smaller kidneys (arrow). (Photos courtesy of Phylonix Pharmaceuticals.)

The worm has turned

The other well-characterized invertebrate model is *C. elegans*, which contains fewer than 1000 somatic cells (*8*). Its simple structure and transparency allow for direct observation of cellular phenotypes (Figure 2). Researchers have extensively studied apoptosis in *C. elegans* and found its regulation to be highly similar to that in mammals (*9*, *10*). Thus, the worm provides a convenient model for studying genes involved in apoptosis and screening for compounds that modulate this process, which has important applications in developing treatments for cancers and neurodegenerative diseases. The nervous system of *C. elegans* is also very simple, making it a good model for studying neurons. NemaRx Pharmaceuticals is using the worm to test drugs for disorders affecting the nervous system, including pain and Alzheimer's disease.

Because of their small size and simple structure, *C. elegans* are very suitable for high-throughput in vivo screenings. Devgen has developed and is using one such system to search for therapeutics for diseases such as diabetes. Union Biometrica has fashioned a particle dispenser useful for automatic manipulation and analysis of *C. elegans*. Researchers have also created imaging algorithms for automated real-time analysis of living worms, enabling high-throughput drug screening based on large-scale behavioral analysis.

Marking mammals

Early-stage animal testing is typically conducted in rodents, followed by drug safety testing and certain efficacy evaluations in larger mammals, such as rabbits and dogs. Technologies for engineering the mouse genome have made it possible to create various disease models for use in screening corresponding therapeutic compounds. Knockout mouse models have been shown to be highly predictive of the effects of drugs that act on target genes. Lexicon Genetics researchers recently created knockout mice for the genes targeted by the top 100 drugs on the market and 100 drugs in pharmaceutical companies' pipelines. They found that phenotypes of these knockout mice correlated highly to the effect of the corresponding drugs (*11*, *12*).

Other techniques for engineering the mouse genome, including knock-in, conditional knockout, and transgenics, have made it possible to create specific gene-sequence alterations and manipulate the levels and patterns of target-gene expression. Using these techniques, researchers can generate specific disease models to validate targets as therapeutic intervention points and screen drug candidates. For example, researchers have generated many mouse models for Huntington's disease by introducing different versions of human Huntington protein fragments carrying expanded polyglutamine repeats (*13*). These transgenic or knock-in mice showed phenotypes characteristic of Huntington's disease patients, including the formation of neuronal inclusion bodies and apoptosis in certain brain regions.

Rodent models are widely used by researchers in the pharmaceutical and biotech industries. To meet this demand, Lexicon Genetics has developed technologies for the high-throughput generation of knockout mice. The technology uses genetically engineered retroviruses that infect mouse embryonic stem cells in vitro, integrate into the chromosome of the cell, and disrupt the function of the gene into which it integrates. Although rodent models are powerful, traditional methods of analysis are slow, relying on ex vivo data collection from tissues removed from sacrificed animals.

Xenogen has developed in vivo biophotonic imaging technologies that enable real-time analysis of drug effects on biological processes in living animals. This allows researchers to collect data for effects on internal organs without the need of surgery, and for time-course effects from the same animals. However, image resolution using this method is relatively low, limiting its application.

Gone fishing

Even with technologies such as biophotonic imaging, using mammals for drug discovery is hampered by high cost and relatively low throughput. Recently, researchers have created assays based on the zebrafish (*Danio rerio*), a small freshwater teleost (*14*). Zebrafish embryos are transparent and develop externally from the mothers, permitting direct assessment of drug effects on internal organs and tissues in vivo (Figure 3). The fish is easy to maintain and breed, and its fecundity is high: Each female can produce 100–200 eggs per mating, providing large numbers of animals for high-throughput assays.

Because of their size, zebrafish embryos and early larvae can be raised in only 100 µL of water in the wells of a 96-well plate for high-throughput whole-animal assays requiring only small amounts of compounds. Drug administration is also simple because researchers can dissolve small-molecule compounds in the water, where they diffuse into the embryos. Alternatively, researchers can microinject larger molecules, such as proteins, directly into the embryos. To knock down specific genes for target validation, morpholino antisense molecules can be injected into one- or two-cell-stage embryos, resulting in uniform distribution of the oligonucleotides across the embryos in several days.

The zebrafish is a highly valid model for studying gene function and drug effects in humans.

Because the zebrafish has most of the same organs found in mammals, it is a much more useful model than *Drosophila* and *C. elegans*. Most human genes have homologues in zebrafish, and the functional domains of proteins, such as ATP-binding domains of kinases, are almost 100% identical between homologous genes, although the similarity over the entire protein is only about 60% (*15*). Because protein function largely resides in functional domains where drugs often bind, the zebrafish is a highly valid model for studying gene function and drug effects in humans.

Indeed, many zebrafish versions of mammalian genes have been cloned and found to have similar functions, and numerous drugs tested in zebrafish caused effects similar to those observed in humans or other mammalian models. For example, research groups have tested anti-angiogenic compounds effective in mammals on zebrafish and found them to have similar effects (*15*, *16*).

The zebrafish is the only vertebrate species for which large-scale forward genetic screens have been carried out, and many mutants obtained from these genetic screens display phenotypes that mimic human disorders, including cardiovascular disease, neurodegeneration, cancer, and blood disease. These mutants not only identify genes that may be involved in diseases but also can be used for drug screening.

Trolling tumorigenesis

Zebrafish are responsive to carcinogenic chemicals and develop neoplasms that are histologically similar to human cancers (*17*, *18*). In addition, because of the rapid development of the zebrafish embryo, researchers can use it for testing drugs that affect cell proliferation. For example, Moon et al. discovered novel microtubule inhibitors by zebrafish embryo screening (*19*).

The zebrafish is also an excellent model for screening for more specific cancer therapeutics, such as angiogenesis and apoptosis modulators. Angiogenesis pathways in zebrafish and mammals are highly conserved, and zebrafish homologues of several important mammalian angiogenesis regulatory genes are expressed in patterns similar to those in mammals. When these genes are mutated or knocked down, they also cause expected effects on angiogenesis (*20*, *21*).

Researchers can easily visualize blood vessels in zebrafish by their endogenous alkaline phosphatase activity, by vessel-specific antibody staining, and, in transgenic zebrafish, with a vessel-specific promoter linked with a reporter, such as green fluorescent protein (*16*, *22*). After the lumen is formed, blood vessels can also be visualized by injecting fluorescent microbeads into the circulatory system as a functional assay to assess the integrity of the vascular system.

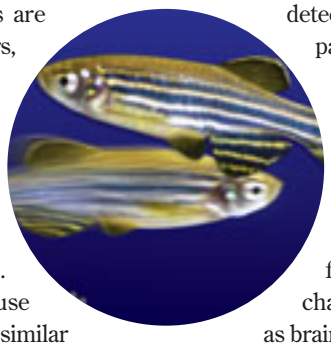
Researchers at Phylonix Pharmaceuticals have tested a number of compounds that showed anti-angiogenic effects in mammals, including SU5416 and flavopiridol. They found that a drug's effect on vessel inhibition in zebrafish correlated well with the effects seen in mammals, indicating that the zebrafish is a predictive model for testing angiogenesis inhibitors (*16*). Because angiogenesis is involved in other diseases, such as diabetic retinopathy and macular degeneration, the angiogenesis assays are also useful for discovering therapies for these diseases.

Angling at apoptosis

Inducing apoptosis is also a promising approach to cancer treatment. The apoptotic processes in zebrafish and mammals are similar, and zebrafish homologues of most mammalian apoptosis-related genes have been identified (*23*). Screening for apoptosis inducers can be performed by looking for their effects in the zebrafish embryo. Researchers can easily detect apoptosis in live embryos by using acridine orange and fluorescence-conjugated caspase sub-

strate (e.g., PhiPhiLuxG1D2). Researchers at Phylonix have developed quantitative assays that can be performed in 96-well microplates in conjunction with a fluorescent plate reader, enabling high-throughput screens (*16*).

Apoptosis is an important mechanism for morphogenesis and homeostasis, and abnormalities in apoptosis are involved in many other diseases in addition to cancers, such as neurodegenerative diseases. Using forward genetic screens, researchers have identified many zebrafish mutants that display abnormal apoptosis, which can serve as models for anti-apoptotic drug screening (*24*).



Researchers can also induce apoptosis in specific populations of cells to mimic certain disorders. For example, aminoglycoside antibiotics can cause degeneration of hair cells in zebrafish neuromasts, similar to the adverse effects of these antibiotics in human hair cells (*25*). The induced hair cell apoptosis can then serve as a model for screening agents to prevent or reverse the damage.

Tackling toxins

Zebrafish and several other teleost species have been used to test environmental toxicants for a long time. Zebrafish-based toxicity assays for drug candidates have also been developed recently. These include assays for organ toxicity, developmental toxicity, and acute toxicity (LC_{50}) (*26*). Changes in organ morphology and the occurrence of necrosis can be directly assessed under a dissecting microscope. Adverse drug effects on cardiac function can be detected by direct observation of heartbeat in the transparent zebrafish embryo (*27*), while other problems, such as neurotoxicity, can be thoroughly examined by staining with cell-type-specific antibodies. Histology for the small zebrafish embryo is simple, because serial sections can be mounted on a single slide and quickly processed for staining.

The zebrafish embryo has been used as a model for studying human fetal alcohol syndrome (FAS). The characteristic features exhibited in human FAS, such as brain defects, are also observed in zebrafish exposed to ethanol (*28*). Teratogenic effects on other organs and structures are also amenable to thorough assessment, making the zebrafish a useful preclinical model for predicting drug toxicity in humans.

With the dramatic rise in the number of potential but poorly validated targets and preliminary hit compounds, small-animal models are increasingly important for validating these targets and profiling the hits. Although several model systems exist, each with its own advantages, zebrafish can bridge the gap between invertebrate and mammalian models. Wider adoption of this small-vertebrate model organism in drug discovery research will help accelerate the drug development process.

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