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Small Zebrafish in a Big Chemical Pond

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ABSTRACT

The number of possible small organic molecules of different structure is virtually limitless. One of the main goals of chemical biologists is to identify, from this ''chemical space'', entities that affect biological processes or systems in a specific manner. This can lead to a better understanding of the regulation and components of various biological machineries, as well as provide insights into efficacious therapeutic targets and drug candidates. However, the challenges confronting chemical biologists are multiple. How do we efficiently identify compounds that possess desirable activities without unwanted off-target effects? Once a candidate compound has been found, how do we determine its mode of action? In this Prospects piece, we call attention to recent studies using embryonic and larval zebrafish to illustrate the breadth and depth of questions in chemical biology that may be addressed using this model, and hope that they can serve as catalysts for future investigational ideas. J. Cell. Biochem. 113: 2208–2216, 2012.

KEY WORDS: IN VIVO CHEMICAL SCREENING; MECHANISM OF ACTION; ANIMAL MODEL

Historically, bioactive chemicals were identified *via* observation of their in vivo phenotypes. This type of discovery process has accounted for many medicines and chemical probes that are used today in clinics and in biological research [Ban, 2006; Gomez-Outes et al., 2011]. However, in the last two decades, in both academia and in the pharmaceutical industry, phenotype-based approaches have been largely replaced by target-based approaches [Lindsay, 2003]. This is partly due to our enhanced understanding of biological systems and disease mechanisms at the molecular level, and partly because in vitro target-based assays can be far more efficient for certain purposes than most in vivo phenotypic assays. Nevertheless, in vitro or cell-based assays are seldom informative about certain important criteria, including specificity, bioavailability, metabolization, and toxicity of a given chemical. In addition, sometimes the molecular target responsible for a physiological function or a disease phenotype is unknown. In such cases, identification of compounds that either elicit or rescue an in vivo phenotype may result in the identification of important molecular targets.

Despite the advantages of in vivo chemical discovery, performing high-throughput screens in mammals can be prohibitively costly. In contrast, zebrafish are fecund, small, and economical. The zebrafish model approximates the best of both worlds when it comes to in vivo models: the tractability of a worm or fly, combined with the physiology of a higher vertebrate.

Chemical screens in zebrafish are in many ways analogous to traditional genetic screens, and they can also be broadly divided into two categories. A conventional screen utilizes wild-type embryos and is used to discover the biological effects and toxicity of test compounds. Occasionally, a close resemblance between the chemical phenotypes and those of zebrafish genetic mutants can be instrumental in determining the mode of action (MOA) of test compounds. On the other hand, a suppressor screen is used to identify compounds that can modulate the phenotypes induced by a genetic, environmental or chemical perturbation that mimics a pathological process. Chemical studies in zebrafish have already identified compounds that are now in clinical trials, have become new research tools, or have identified the MOA of an existing drug [Zhang et al., 2006; North et al., 2007; Yu et al., 2008b]. Understanding the strengths and limitations of this model organism will likely open further doors to new ideas and promising research directions.

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FEASIBILITY OF CHEMICAL SCREENING AND PROPERTIES OF COMPOUNDS IDENTIFIED IN ZEBRAFISH

Embryonic/larval zebrafish between 0 and 5 days post-fertilization (dpf) are permeable to small molecules and compact enough for up to 10 embryos to fit into a well of a 96-well plate, making them suitable for high-throughput chemical screening. Zebrafish develop rapidly, and within 24 hpf, an embryo has already developed a circulatory system and many major organs, such as eyes, ears, and a central nervous system. By 5 dpf, a digestive system, pronephric kidney, and definitive hematopoietic cell lineages have all been established. Thus, using embryonic/larval zebrafish, chemical effects on multiple biological processes may be detected simultaneously in a reasonably short time-frame. In addition, zebrafish embryos are transparent, enabling easy visualization of their internal organs (Fig. 1).

The first pioneering chemical screen in zebrafish demonstrated that compounds added into the surrounding water can penetrate the embryos and that their effects on the development of various physiological systems can be readily identified by visual inspection of the treated embryos [Peterson et al., 2000]. A follow-up larger-scale developmental screen revealed the general properties of compounds that exhibited bioactivity in zebrafish [Sachidanandan et al., 2008]. The authors showed that all of the bioactive compounds identified in this study were hydrophobic, as they had positive logP (octanol:water partition coefficient) values. In addition, many of the bioactive compounds had molecular weights between 200 and 500 kDa. These results suggest that compounds active in zebrafish exhibit certain physicochemical properties common to most oral drug candidates [Lipinski et al., 2001]. Together, these screens illustrate the use of the zebrafish for the identification of novel compounds that have no previously designated bioactivity. Notably, some of the compounds identified in a seemingly rudimentary screen exhibit surprisingly specific effects, in that the compounds can elicit a specific phenotype over a wide range of concentrations (1000-fold range) without inducing toxicity or additional phenotypes [Peterson et al., 2000].

IDENTIFICATION OF NOVEL BIOACTIVE COMPOUNDS THAT AFFECT SPECIFIC PHYSIOLOGICAL PROCESSES

A more focused screen may use either a fluorescent reporter zebrafish line or a staining procedure to mark a specific cell type of interest. These detection methods increase the sensitivity of the screens, and allow for assay methods to be uniquely adapted to the functionality of a specific cell type. In contrast to in vitro assays, a developing embryo offers a native physiological context and

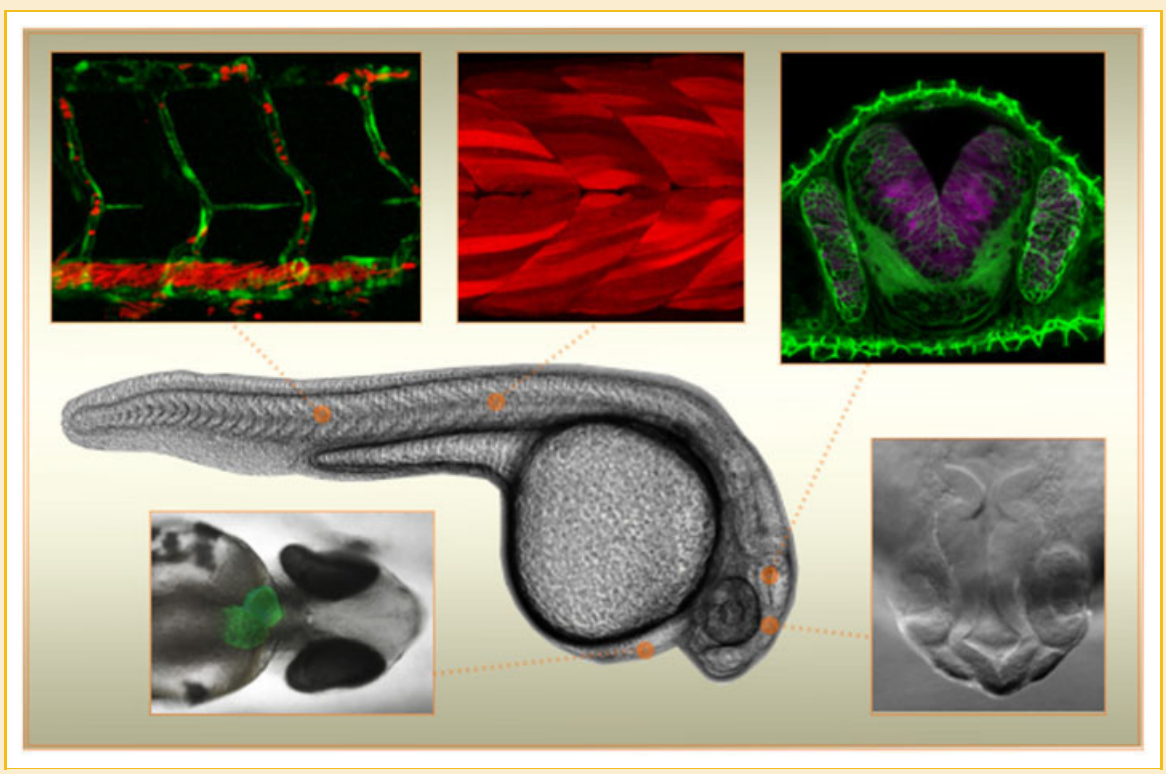

Fig. 1. Rapid development and easy visualization of zebrafish embryos. A 1-day-old zebrafish embryo (center) has already developed multiple organ systems, which can be easily observed due to transparency of the embryos and using fluorescent transgenic lines to highlight specific compartments. The effects of small molecules on the morphology or function of multiple tissues can thus be readily detected. *Panels*: *kdrl*-GFP and *gata1*-DsRed show individual DsRed^{+} red blood cells within GFP^{+} vessels (top left); *mylz2*-DsRed shows muscle cells and myotome structure (top middle); membrane-targeted GFP and an mCherry enhancer trap strain show structure of the brain (top right); embryonic heart expressing *cmlc2*-GFP in cardiomyocytes (bottom left); bright-field image of the embryonic brain ventricle (bottom right).

enables the identification of compounds that affect various aspects of cell behaviors, such as differentiation, proliferation, and migration. Furthermore, complex developmental processes, such as organ formation and physiological responses, such as behaviors cannot be modeled in vitro. We will discuss a few examples below to exemplify the diversity of physiological processes that may be studied in zebrafish in vivo.

HEMATOPOIETIC STEM CELLS

Compounds that promote the development of hematopoietic stem cells (HSCs) may be used clinically to enhance the efficiency of bone marrow transplantation. However, culturing HSCs in vitro is quite difficult, expensive, and inefficient. North et al. [2007] screened a panel of known bioactive compounds to identify the ones that could affect HSC numbers during embryonic development. Using in situ hybridization of *runx1* and *cmyb*, two genes expressed in emerging HSCs in both zebrafish and mammals, the authors successfully identified a series of compounds that either enhanced or reduced HSC numbers. Grouping these compounds by their known bioactivities revealed regulators of prostaglandin E2 (PGE2) synthesis as regulators of HSC development in vivo. Subsequent experiments confirmed that PGE2 could also increase the number of hematopoietic progenitors in mouse embryonic stem cell differentiation assays as well as the number of HSCs in bone marrow transplants after transient ex vivo exposure. The findings from this study have not only led to a clinical trial using PGE2 during bone marrow transplantation, but also sparked a number of follow-up studies that shed light onto other important factors that regulate HSC development [North et al., 2009; Goessling et al., 2011].

CARDIAC PROGENITORS

Agents that induce the growth of cardiac progenitors are sought-after for their potential to stimulate heart regeneration in ischemic hearts or after heart attacks. While several compounds have been shown to promote the growth of cardiac progenitors in cell culture, recently a developmental screen in zebrafish was performed to identify compounds that could increase heart size. Using a fluorescent cardiomyocyte reporter line, Ni et al. [2011] quickly identified a class of compounds that could increase heart size by expanding the numbers of cardiac progenitors. The researchers showed that this class of compounds, which they called cardiogenins, potently induced differentiation of mouse embryonic stem cells into cardiac progenitors, suggesting that cardiogenins could target an analogous pathway in mammals. The authors also showed that cardiogenins induced a rather specific heart phenotype, while other compounds appeared to have less heart-specific activities [Ni et al., 2011]. This highlights the utility of the zebrafish in allowing investigators to simultaneously detect phenotype-of-interest as well as any unwanted effects.

ANGIOGENESIS

Angiogenesis or neovascularization is a complex process involving endothelial cells and their response to environmental cues. Anti-angiogenic compounds are presumptive anti-cancer agents, whereas angiogenic compounds are potentially beneficial in cases of ischemia and wound healing. Numerous studies have taken advantage of several fluorescent endothelial cell reporter lines in zebrafish for the identification of novel anti-angiogenic compounds (reviewed in [Hasso and Chan, 2011]; Fig. 1).

PANCREATIC β-CELLS

Regenerating β-cell mass in diabetic patients is currently an area under intensive investigation. Insulin-producing cells are formed in two waves during zebrafish development, but only β-cells of the secondary islets contribute to the adult endocrine pancreas [Hesselson et al., 2009]. Thus, it has been hypothesized that compounds capable of inducing secondary islet formation may be therapeutic candidates for β-cell regeneration. It is known that inhibition of Notch signaling can induce precocious formation of secondary islets in zebrafish, but Notch inhibitors are probably unsuitable therapeutic candidates for regenerative medicine due to their potential side effects [Rovira et al., 2011]. To identify compounds targeting other pathways, Rovira et al. [2011] screened a compound library consisting of mainly FDA-approved drugs using zebrafish that carried a Notch-responsive *mCherry* reporter and a *pax6b-GFP* reporter marking pancreatic endocrine cells. The authors identified a few classes of compounds that induced GFP-positive cells without reducing *mCherry* expression. They further characterized two compounds, mycophenolic acid (MPA) and disulfiram (DSF), and found that these clinically available drugs could also induce β-cell differentiation from human pancreatic duct carcinoma cells.

DIGESTIVE SYSTEM

Larval zebrafish usually start to ingest food at 5 dpf, since this is when their yolk is mostly consumed and their digestive system is fully developed. Several fluorescently labeled fatty acid and cholesterol probes have been developed that can be used for investigating digestive function and lipid metabolism in zebrafish [Hama et al., 2009; Clifton et al., 2010; Carten et al., 2011]. Some of these probes are quenched fluorescent reporters that can be activated by intestinal enzymes [Hama et al., 2009]. Using these various probes, a chemical screen has been carried out in 5 dpf zebrafish to identify compounds that could potentially interfere with dietary lipid absorption [Clifton et al., 2010]. In addition to detecting digestive and metabolic function, these probes may be used to image changes in morphology of the digestive organs as the potential outcome of drug effects [Carten et al., 2011].

NEUROLOGICAL RESPONSES AND BEHAVIOR

Attracted by the feasibility of performing a large-scale chemical or genetic screen in zebrafish, many labs have started to develop automated behavioral assays in zebrafish [Kokel et al., 2010; Rihel et al., 2010; Wolman et al., 2011]. To date, two large- and one medium-scale behavioral chemical screens have been conducted in zebrafish, all in very young animals (1–5 dpf). Rihel et al. [2010] developed an automated assay for measuring the motion of fish during the sleep/wake cycle in 96-well plates and identified several therapeutic compounds that could affect waking activities at night or during the day. Wolman et al. [2011] showed that zebrafish larvae

exhibit non-associative learning ability and uncovered a set of novel chemical cognitive modulators.

Another screen utilized a novel photomotor response that was identified in embryonic zebrafish and characterized by Kokel et al. [2010]. The photomotor response is a series of very distinct motion profiles in response to light stimuli. Using this series of profiles as "behavioral barcodes," the authors found that different classes of psychotropic compounds elicited different and unique patterns of barcodes. Thus, based on the in vivo phenotypes that some compounds induced, they were able to classify several new neuroactive small molecules. Interestingly, one of the acetylcholinesterase inhibitors that they found is in fact a pro-drug (a compound that needs to be metabolized in vivo to become bioactive), which would not have been discovered using in vitro or cell-based assays.

MODE OF ACTION (MOA) STUDIES OF BIOACTIVE COMPOUNDS

Identifying the MOA of a bioactive compound is a critical step in deriving a better drug or in realizing its utility as a new chemical probe. However, moving from initial compound identification to defining its MOA can pose a substantial technical hurdle. Compounds may be associated with specific molecular targets through the power of deduction based on genetic pathways known to affect a given phenotype. For example, in a chemical screen, DTAB was found to modulate anterior–posterior patterning of zebrafish embryos [Sachidanandan et al., 2008]. A similar phenotype had been previously observed in genetic mutants that resulted in overproduction of retinoids [Diez del Corral et al., 2003]. This connection in their phenotypes led to a simple set of in vitro experiments confirming that DTAB is indeed an agonist of retinoic acid receptors [Sachidanandan et al., 2008]. The pathway targeted by the above-mentioned cardiogenins was also arrived at by deduction from previously documented mutant phenotypes [Ni et al., 2011]. Similarly, phenotype clustering with known bioactive compounds may reveal MOA of new compounds, as shown in the photomotor response study [Kokel et al., 2010]. In these examples, the compounds in question were originally identified in zebrafish screens and their phenotypes immediately called attention to their MOA.

Another example is the anti-angiogenic drug TNP-470 that is currently in clinical trials under the name Lodamin. TNP-470 is a synthetic analog of the fungal metabolite fumagillin. The cellular target of fumagillin, methionine aminopeptidase (MetAP-2), was identified through biochemical purification [Sin et al., 1997]. Although gene targeting in mice confirmed that MetAP-2 played a critical role in angiogenesis during embryonic development, it was difficult to dissect the molecular pathway downstream of MetAP-2 using the mouse model [Yeh et al., 2006]. Interestingly, a follow-up zebrafish study showed that both genetic knockdown of MetAP-2 and chemical treatment with fumagillin phenocopied a truncated posterior defect of the *wnt5a* mutants [Zhang et al., 2006]. This finding has led to the identification of the first non-canonical Wnt inhibitor and a role for non-canonical Wnt signaling in endothelial cell growth. It is worth noting that chemical MOA studies in zebrafish benefit from a valuable database on the zebrafish research community website ZFIN (http://www.zfin.org). This database compiles all published results linking genotype to phenotype, to which chemotype can then be compared.

IDENTIFICATION OF PATHWAY-SPECIFIC CHEMICAL MODULATORS

The genotype–phenotype archive of zebrafish may also be useful for purposefully finding bioactive compounds targeting specific signaling pathways. A good example is a chemical screen in zebrafish that uncovered one of the first BMP antagonists [Yu et al., 2008b]. While many conserved signaling pathways share similar frameworks, subtle differences in individual pathway components or their structures can be enough to confer distinct biological interactions or functions. For example, signal transduction of both transforming growth factor β (TGF-β) and bone morphogenetic protein (BMP) utilizes unique but closely related receptors, and these two pathways share some downstream components, yet they often play very different roles in vivo. Thus, in vitro screens based on purified TGF-β or BMP receptors may not distinguish chemicals that will effectively modulate each specific pathway. While TGF-β signaling is involved in the establishment of axial patterning (mutants may be cyclops), BMP signaling is involved in dorsoventral patterning (mutants may be shaped like American footballs instead of soccer balls) [Kishimoto et al., 1997; Feldman et al., 1998; Sampath et al., 1998]. Based on a BMP receptor mutant phenotype, Yu et al. [2008b] performed a straightforward developmental screen and identified a compound named dorsomorphin that caused a similar dorsalization phenotype. Subsequently, the authors showed that dorsomorphin selectively inhibits the BMP type I receptors but not TGF-β receptors. Only 4 years since its identification, dorsomorphin has been used in more than 40 publications as a specific BMP antagonist. Dorsomorphin and its derivatives have shown efficacy in various disease models where the human disease has been linked to hyperactive BMP signaling, such as fibrodysplasia ossificans progressiva (FOP) [Yu et al., 2008a].

Thus, in some cases, there may be important benefits to taking an in vivo versus in vitro target-based approach. For example, a significant obstacle in target-based drug design is the structural similarity between members of a protein family, such as kinases or G protein-coupled receptors. A kinase or a receptor may be subject to different post-translational modifications or protein–protein interactions depending on the cellular context, so an in vitro assay may not faithfully represent the state of the target protein. If some of the family members play non-redundant roles in vivo, their mutant phenotypes can be a good indicator for finding chemical inhibitors that exhibit in vivo efficacy and specificity.

STRUCTURE–ACTIVITY RELATIONSHIP (SAR) STUDIES

After the initial identification of a bioactive compound, SAR studies are often performed in order to identify analogs that are more potent, more specific, and more easily synthesized. The initial stages

of SAR studies are usually performed using in vitro assays against, whenever possible, purified molecular targets. In drug development, additional essential parameters that need to be considered for deriving a better drug candidate are absorption, distribution, metabolism, excretion, and toxicology (referred to as ADMET). These parameters determine how a compound behaves in vivo and therefore an in vivo model is often used for these studies. However, due to the high costs associated with many in vivo models, such as mice and primates, usually only limited numbers of selected compounds are tested in the animal. In a recent study, Hao et al. [2010] screened 63 dorsomorphin analogs in a zebrafish assay. Previously, it was found that dorsomorphin induced not only a dorsalization phenotype but also an angiogenesis defect [Yu et al., 2008b], and it had been postulated that BMP signaling might play a role in angiogenesis. By altering the functional groups around the dorsomorphin backbone, the investigators found that these two in vivo phenotypes could be decoupled [Hao et al., 2010]. These results suggested that the two phenotypes were mediated via two independent molecular targets, possibly BMP and vascular endothelial growth factor (VEGF) receptors. This hypothesis was later confirmed using in vitro kinase assays. In this study, the authors noted that some of the compounds showed discrepancy between their in vitro and in vivo activities, which might be due to their low in vivo bioavailability. Thus, incorporating zebrafish assays into SAR studies may provide significant cost benefits and identify better drug candidates at the early stages of drug development. Another advantage of conducting SAR studies on small molecules in vivo is the ability to detect off-target effects simultaneously.

TOXICOLOGY STUDIES

It is not difficult to understand the interest in using zebrafish for teratology and toxicology studies: test compounds can be administered directly into the water, and tiny perturbations in myriad signaling pathways may cause observable detrimental effects on developing embryos. For public health reasons, there are tens of thousands of compounds worldwide that require safety assessment. The U.S. Environmental Protection Agency recently added a zebrafish developmental assay to its Computational Toxicology Research Program to help elucidate the toxicity of potentially hazardous chemicals [Padilla et al., 2011]. Currently, this assay is only based on death and overt structural defects but could be expanded to provide more detailed information, for example, on endocrine disruption, immune modulation, or genotoxicity [Scholz et al., 2008].

Can zebrafish be used for preclinical toxicology (tox) tests? Compared to humans, zebrafish possess several analogous physiological systems, with the notable exceptions of placentation and the lungs. Many studies have shown that zebrafish exhibit not only molecular and physiological similarities to humans, but also comparable pharmacological responses. For example, a common cause of drug withdrawal from the market is inhibition of the hERG channel, leading to potentially lethal arrhythmia. Milan et al. [2003] showed that, of 23 compounds that were known to induce arrhythmia in humans, 22 compounds also caused arrhythmia in zebrafish. Antisense knockdown of the hERG ortholog in zebrafish caused a similar phenotype, indicating that the zebrafish is a valid model for identifying drug interactions with the hERG channel [Milan et al., 2003]. Long-term use of aminoglycoside antibiotics has been linked to hearing loss in humans, and these antibiotics also cause mechanosensory hair cell death in zebrafish [Owens et al., 2008]. Overdoses of the antianalgesic and antipyretic medication acetaminophen can cause acute liver failure in humans and similar hepatotoxicity in zebrafish [North et al., 2010]. Further, one of the side effects associated with the heavily prescribed cholesterol-lowering statins is muscle pain or breakdown. Consistent with this, statins induce myopathy in zebrafish [Hanai et al., 2007].

For specific preclinical tox screening, adult zebrafish may be used for some types of blood tests or tissue extraction. However, embryonic zebrafish may be very useful for detecting drug-induced effects on cardiac functionality, cell death, metabolic changes, expressional profiles, organ structures, and even animal behaviors. Due to the ease of in vivo imaging and ability to develop versatile, high-throughput assays, zebrafish hold great promise for preclinical tox screening. Several biotech companies are already offering tox screening services and some drug companies now even have in-house zebrafish tox screening projects. However, the true value of zebrafish models in predicting drug safety will only be realized after more data have been collected.

CHEMICAL SUPPRESSOR SCREENS

A recent survey of all the small-molecule first-in-class drugs approved by the FDA from 1999 to 2008 showed an interesting result. The study investigated the strategies used to discover these drugs and found that 60% were originally identified by phenotypic screening [Swinney and Anthony, 2011]. This finding is somewhat surprising considering the emphasis on target-based approaches in most pharmaceutical companies in recent years. Target-based approaches identify compounds that alter the activity of a specific molecular target. These compounds may not reverse a disease phenotype, or they may possess unexpected biological effects [Ban, 2006]. In contrast, phenotype-based chemical suppressor screens identify compounds by their ability to suppress a disease phenotype rather than their ability to modulate a specific molecular target.

Complex diseases that cannot be modeled either in vitro or in a cell-based system may be modeled in an in vivo system such as zebrafish. Studies combining zebrafish models of human disease and chemical screening have contributed to our understanding of many disease areas and identified potential therapeutic candidates. Some of these are discussed below.

CHEMICAL SUPPRESSORS OF AORTIC COARCTATION

This was the very first chemical suppressor screen in zebrafish. Coarctation (narrowing) of the aorta is a congenital birth defect in humans. The genetic determinants of this condition are unclear, but it is mostly treatable by surgery. Interestingly, zebrafish *gridlock* mutant embryos manifest a phenotype similar to human aortic coarctation, where no blood circulates to the trunk region. Peterson

et al. [2004] identified a class of compounds with unknown bioactivities that restored blood flow in mutant embryos. Transcriptional profiling analysis showed that these compounds induced VEGF expression in both fish embryos and human umbilical vein endothelial cells (HUVECs). Subsequently, the same group performed another screen with a similar scheme but a different chemical library, and identified another class of gridlock suppressors [Hong et al., 2006]. The authors found that these two distinct classes of gridlock suppressors target two nodal points of one signaling pathway, and concluded that both activation of VEGF signaling and inhibition of PI3K activity are essential for the arterial specification of endothelial cells.

CHEMICAL SUPPRESSORS OF A LEUKEMIA ONCOGENE AML1-ETO

Acute myelogenous leukemia (AML) is the most common form of adult leukemia worldwide, with about 107,000 new cases reported annually [Stone et al., 2004]. The chromosomal translocation that results in the *AML1-ETO* (*AE*) fusion oncogene is found in 12–15% of AML patients. These patients often exhibit an overabundance of granulocytic blast cells in the blood and bone marrow. Despite extensive studies of AE's function in cells and in mice, targeted therapy against AE's oncogenic effects remains unavailable and thus we decided to study AE's signaling pathways in zebrafish.

During zebrafish development, myelopoiesis and erythropoiesis take place in two distinct intraembryonic domains (reviewed in [Chen and Zon, 2009]). These two pools of blood cells offer a unique opportunity for analyzing transcriptional regulation governing myeloid and erythroid differentiation. By analyzing expression of hematopoietic genes in situ at various developmental stages (12–48 hpf), we found that expression of AE converts erythropoiesis to myelopoiesis in multipotent hematopoietic progenitors. Subsequently, AE promotes granulocytic differentiation while inhibiting their maturation, resulting in the accumulation of immature granulocytic blast cells, as observed in human patients [Yeh et al., 2008]. Using a chemical suppressor screen, we found that inhibitors of the cyclooxygenases (COXs) could reverse AE's phenotype [Yeh et al., 2009]. Based on this finding, we further showed that AE induced COX-2 expression, which led to the activation of β-catenin-dependent signaling in zebrafish, human leukemia cells, and mouse bone marrow cells [Yeh et al., 2009; unpublished results]. We then investigated the role of COX-2/β-catenin signaling in AE's oncogenic effects. From our published and unpublished results, we propose that COX inhibitors such as non-steroidal anti-inflammatory drugs (NSAIDs) may provide therapeutic benefits for patients with various subtypes of AML.

It is known that, in cancer cells, embryonic signaling pathways are sometimes re-activated [Takebe et al., 2011]. Our studies suggest that examining AE's effects in the embryonic zebrafish model identifies a conserved signaling pathway in AE-mediated leukemogenesis. Another study showed that chemicals that suppress embryonic development of neural crest cells, which are the precursors of melanocytes, can exhibit significant inhibitory effects on xenograft tumors of human melanoma cells [White et al., 2011]. In concert, these results indicate that several types of cancer cells rely on signaling pathways utilized by embryonic progenitor cells, and that embryonic zebrafish models may be used for the identification of these critical pathways.

CHEMICAL SUPPRESSORS OF LONG QT SYNDROME

Human long QT syndrome is a disorder of prolonged cardiac repolarization, which can lead to sudden death due to uncontrollable arrhythmia. Zebrafish *breakdance* mutants carry a mutation (I59S) in the zebrafish ortholog of the hERG gene, which has been implicated in type 2 long QT syndrome [Peal et al., 2011]. This mutant recapitulates the prolongation of cardiac repolarization, resulting in a 2:1 atrioventricular block phenotype (the ventricle beats once after the atrium beats twice), easily detectable in transparent embryos. Based on this phenotype, Peal et al. performed a chemical suppressor screen and identified two lead compounds that were able to reverse the *breakdance* phenotype. The authors found that one of the compounds, flurandrenolide, functions via a glucocorticoid receptor-dependent mechanism, while the MOA of the other compound, 2-MMB, is completely unknown. These two compounds also partially rescued repolarization defects in mammalian cells. As a result of these findings, the glucocorticoid steroid dexamethasone is being investigated in a clinical trial for long QT syndrome.

HEPATOPROTECTIVE AGENTS OF ACETAMINOPHEN-INDUCED INJURY

Chemical suppressor screens may be used to identify protective agents even when the phenotype is caused by a chemical insult and not by a genetic defect. Acute hepatotoxicity is a serious clinical concern resulting from acetaminophen (APAP) painkiller overdose. North et al. [2010] showed that treatment of 2–4 dpf zebrafish with a high dose of APAP causes acute liver injury similar to that in humans. Using a fluorescent liver reporter line, the authors tested 96 preselected compounds and found that PGE2 could restore liver size after APAP treatment. Although the mechanisms by which PGE2 may protect from APAP-induced liver injury are still under debate, the study demonstrates proof of concept that the zebrafish model can be used to identify suppressors of chemotoxicity.

CHEMICAL SUPPRESSORS OF DUCHENNE MUSCULAR DYSTROPHY

Mutations in the human dystrophin gene are the cause of Duchenne muscular dystrophy (DMD). Previously, genetic screens in zebrafish identified two mutant lines, *sapje* and *sapje-like*, that carried null mutations of the dystrophin gene. These mutants exhibit a lethal muscle degeneration phenotype between 2 and 5 dpf, which can be detected using a birefringence imaging method [Kawahara et al., 2011]. Placing zebrafish embryos between two polarizing filters reveals a regular pattern of normal muscle segments, or an irregular pattern of degenerated muscle segments. A chemical suppressor screen identified a number of chemicals that not only reversed the mutant muscle patterning phenotype, but also extended survival of the mutant zebrafish [Kawahara et al., 2011]. There are two important findings from this study. First, none of the compounds identified in the screen restored dystrophin expression, suggesting that there are additional therapeutic targets for DMD and that they may play a role in other types of muscle degenerative diseases.

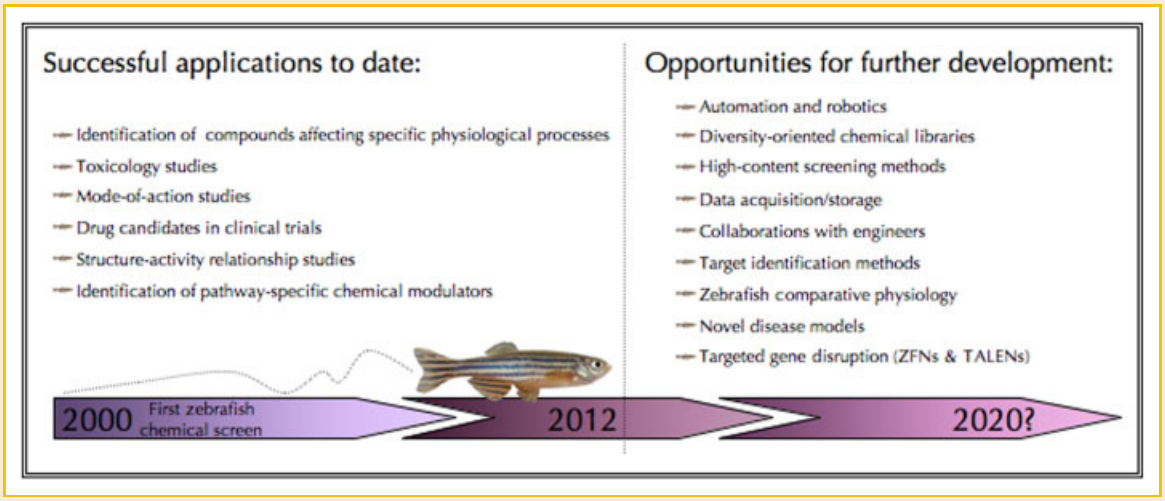

Fig. 2. Progress of chemical biology in the zebrafish. Since the first zebrafish chemical screen in 2000, the zebrafish model has been successfully applied to several questions in chemical biology. Further advantage could be taken of the model in the future by incorporating a number of improvements. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Second, at least two FDA-approved drugs, aminophylline and sildenafil (Viagra), may be potential therapeutic agents for DMD.

SUMMARY

The addition of zebrafish to the chemical biologist's toolbox has made salient contributions towards tackling various challenges in this field, enabling innovative and effective methods for chemical screening, MOA, SAR, and toxicology studies. The works outlined here underscore the value of incorporating in vivo phenotyping in order to faithfully assess the effects of bioactive chemicals.

Chemical screening or testing in zebrafish is not intended to completely replace in vitro or cell-based assays. Importantly, target-based approaches enable knowledge-driven drug design and eliminate the lengthy process of identifying the molecular target of a candidate compound. However, in vivo assays are especially valuable when the molecular mechanism underlying a biological phenomenon is unclear or when the cause of a disease cannot be addressed directly, as in the case of undruggable oncoproteins or certain genetic diseases. In addition, instead of one target at a time, in vivo screening can test all potential targets in a biological system at once. In support of such non-reductionist approaches, several drugs approved by the FDA in the last 10+ years were identified by phenotypic screening [Swinney and Anthony, 2011].

In the future, to expand the utility of the zebrafish, we need to gain a better understanding of the biological aspects of this model that are currently incompletely understood, and to determine the exact extent of the physiological and pharmacological similarities between fish and humans. Clearly, studies to date have performed very well at the cellular and organ levels (Fig. 2). However, studies on systemic-level processes, such as aspects of metabolism or the immune response, have been relatively limited. The zebrafish model may also be used for studying unique biological phenomena not available in a mammalian model, such as tissue regeneration. Therefore, opportunities exist for the development of novel assays that have the capability of surveying a wider range of phenotypes and biological processes.

Recently, we and others have successfully implanted zinc finger nuclease (ZFN) and transcription activator-like effector nuclease (TALEN) technology into zebrafish for performing targeted gene disruption [Sander et al., 2011ab]. Together with transgenesis, cre-lox, and various inducible systems available in the zebrafish model, these genomic engineering tools will likely accelerate the development of models of human disease in the zebrafish.

A majority of the chemical screens conducted to date in zebrafish are of medium scale (~2,000 compounds) using chemical libraries enriched in known bioactive compounds or FDA-approved drugs. The rationale for choosing these libraries is to increase the hit rate, bypass the need for target identification, and to increase the likelihood of successfully translating a discovery from bench to bedside. Most labs will be able to perform a screen at such a scale even without any automation. Further refinement of current automation, robotic handling, and data acquisition/storage methods through collaborations with engineers will enable the screening of larger libraries of greater structural diversity in higher throughput [Pardo-Martin et al., 2010; Graf et al., 2011; Walker et al., 2012]. With these lessons in hand, we may more boldly and effectively explore unknown chemical space in the years ahead.

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