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Cancer and Zebrafish: Mechanisms, Techniques, and Models: Chemical Genetics

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Abstract

Chemical genetics is the use of small molecules to perturb biological pathways. This technique is a powerful tool for implicating genes and pathways in developmental programs and disease, and simultaneously provides a platform for the discovery of novel therapeutics. The zebrafish is an advantageous model for *in vivo* high-throughput small molecule screening due to translational appeal, high fecundity, and a unique set of developmental characteristics that support genetic manipulation, chemical treatment, and phenotype detection. Chemical genetic screens in zebrafish can identify hit compounds that target oncogenic processes— including cancer initiation and maintenance, metastasis, and angiogenesis—and may serve as cancer therapies. Notably, by combining drug discovery and animal testing, *in vivo* screening of small molecules in zebrafish has enabled rapid translation of hit anti-cancer compounds to the clinic, especially through the repurposing of FDA-approved drugs. Future technological advancements in automation and high-powered imaging, as well as the development and characterization of new mutant and transgenic lines, will expand the scope of chemical genetics in zebrafish.

Keywords

chemical screen; chemical genetics; zebrafish; cancer; drug discovery; therapeutics

1. Introduction

Chemical genetics is the use of small molecules to perturb biological pathways. Target-based and phenotype-driven chemical screens can elucidate mechanisms of developmental programs and signal transduction pathways, and simultaneously identify novel therapeutic drugs. Chemical screens may be designed with a target-based approach, in which libraries of compounds are assayed *in vitro* for activation or inhibition of a known molecular target. Chemical hits identified to act on a specific target can then be evaluated *in vivo*. In a phenotype-driven chemical screen, small molecules are used to disrupt signal transduction pathways *in vitro* or *in vivo*, causing aberrant phenotypes. Targets of chemical hits can then be identified using biochemical and chemoinformatic techniques. Forward phenotype-driven genetic screening of large small molecule libraries is a powerful tool for implicating previously unidentified genes and pathways in disease.

While cell-based and biochemical small molecule screens have proven successful in identifying novel therapeutics, these methods often fail *in vivo* validation. For example, compounds can induce a quantifiable response *in vitro*, but are highly toxic or possess poor pharmacological properties *in vivo*. Similarly, compounds may fail to elicit a detectable response *in vitro*, but cause a significant, robust response *in vivo* due to the presence of niche microenvironments or complex physiological signaling networks within the organism. For these reasons, the use of *in vivo* animal models enables more precise detection of physiologically relevant events and, in parallel, evaluations of drug efficacy and toxicity.

The zebrafish, *Danio rerio*, has been established as an indispensable *in vivo* model for chemical genetics and offers many benefits over traditional *in vitro* screening approaches. Genetic conservation of biological pathways between zebrafish and humans promotes the identification of clinically relevant drugs. High fecundity makes the zebrafish amenable to large-scale chemical screening, with females producing up to 300 embryos per week. Embryos develop *ex utero* on a scale of days, allowing for rapid screening. The aqueous environment of development is well-suited for chemical solubilization for high-throughput drug delivery and offers temporal control of pathway perturbation. Finally, embryo transparency enables visualization of all developmental stages and facilitates the detection of aberrant phenotypes. Taken together, the zebrafish has many advantages that can be scaled and optimized for chemical genetic screening.

The first high-throughput forward chemical genetic screen assayed 1,100 synthetic small molecules to investigate vertebrate development and identify novel compounds that regulate embryogenesis (Peterson *et al.*, 2000). Most importantly, the use of whole zebrafish embryos enabled researchers to simultaneously assess the effect of each small molecule on all developmental pathways. The screen identified several compounds that modulate development of the central nervous system, the cardiovascular system, the neural crest, and the ear (Peterson *et al.*, 2000). Since this pioneering study, *in vivo* chemical screens using zebrafish have been utilized to study vertebrate development (Moon *et al.*, 2002 and Khersonsky *et al.*, 2003 and Mathew *et al.*, 2007 and Sachidanandan *et al.*, 2008), behavior (Kokel *et al.*, 2010), and disease (Peterson *et al.*, 2004 and Shepard *et al.*, 2005 and Peal *et al.*, 2011 and Zhang *et al.*, 2013). Notably, *in vivo* chemical screening using zebrafish has been instrumental in identifying novel cancer therapeutics (Wang *et al.*, 2010 and White *et al.*, 2011 and Ridges *et al.*, 2012 and Le *et al.*, 2013 and Gutierrez *et al.*, 2014 and Astin *et al.*, 2014).

This chapter outlines the general principles of chemical screening, with a focus on screen design, advantages of the zebrafish system, and future technologies to overcome current limitations. In addition, we highlight the important contribution of chemical genetics to cancer research, profiling three chemical screens performed in zebrafish to identify genes involved in oncogenic pathways and to discover novel melanoma and T-cell acute lymphoblastic leukemia (T-ALL) therapies.

2. Chemical Genetic Screens

2.1. Experimental Workflow

The workflow of *in vivo* small molecule screens using zebrafish embryos (Figure 1) is straightforward and highly customizable to the scientific investigation. A small-scale trial screen is first performed with a limited number of embryos and compounds to optimize experimental parameters. The number of embryos, choice of chemical library, timing and duration of chemical exposure, chemical concentration, and embryonic stage are important variables that require optimization (Kaufman et al., 2010 and Peterson et al., 2011 and Tan et al., 2011, Rennekamp et al., 2015).

In a full-scale screen, a large number of viable, developmentally synchronous embryos are first collected. This can be achieved through the setup of numerous individual breeding cages, typically containing 5 fish: 3 females per 2 males. Alternately, up to 200 adult zebrafish fish can be bred *en masse* using a specialized breeding vessel, producing up to 10,000 developmentally synchronous embryos within minutes (Adatto *et al.*, 2011). Viable embryos are selected and re-distributed into individual wells of a transparent multi-well plate. Plate format ranges from 12- to 1536-well, with 96-well being the most commonly used.

Embryo array is followed by chemical exposure, and parameters to consider include choice of chemical library, timing and duration of exposure, chemical concentration, and general toxicity. Chemicals libraries range in size from 10 to 50,000 compounds and come in three varieties: commercial vendor, natural product, and synthetic. The most commonly used libraries are the LOPAC collection of bioactives and the Chembridge DIVERSetE collection of synthetic compounds. Bioactive libraries, a subset of commercial vendor libraries, offer the advantage of well-annotated chemicals and pathway/protein targets, which are helpful in determining the activity of a compound. In general, compounds are added no earlier than 6 hours post-fertilization (hpf), also known as the 50% epiboly stage, and removed within 24 hours to minimize toxicity. Chemicals must be added when a specific targeted process or biological pathway is active, so understanding the developmental context is essential when designing the window of chemical exposure. Compound concentrations range from 1 to 100 mM, with 10 and 20 mM dilutions being the most frequently assayed. Compounds can be added to wells by hand for small-scale screens or using a liquid-dispensing robot, such as the TECAN robot (Tecan, Durham, NC) or Sciclone ALH3000 liquid robot handler (Caliper Life Sciences, Waltham, MA), for large-scale screens.

Following chemical exposure, embryos are evaluated for phenotypic alterations, which can include morphological abnormalities and complex molecular changes detectable via fluorescence imaging or secondary biochemical assays. Typically, chemical screens investigating oncogenic pathways have utilized transgenic zebrafish lines and detected chemical hits by scoring embryos for alterations in cell state. Assessing changes in cell state requires a secondary assay, such as *in situ* hybridization (ISH) to detect RNA expression or immunohistochemistry to detect protein expression. Fluorescence-based microscopy may be used to observe reporter genes.

Chemical hits are re-screened for validation, often to assess optimal concentrations via dose responses. Validated hits are further studied to identify protein targets and mechanisms of action, using the chemical structure and binding profiles, if available. Candidate-based identification has been the favored approach and typically involves the use of annotated bioactive libraries, chemoinformatic analysis, and/or genetic studies. Annotated bioactive libraries are frequently used for screening because they contain information about the pathway and protein targets of small molecules. If the target of a small molecule is unknown, chemoinformatic analysis can be employed to identify structurally similar compounds with known activities. Alternately, the chemical of interest can be tagged with a magnetic bead, such that its endogenous bioactivity is unaltered, and isolated for the identification of binding partners via biochemical assays and computer simulations. Finally, genetic studies can be used to elucidate the activity of a compound. For example, gene expression can be measured by microarray analysis, real-time PCR (RT-PCR), or ISH, while candidate genes can be knocked down in an attempt to phenocopy the effect of the small molecule.

2.2. Advantages of *in vivo* small molecule screening using a zebrafish model

The zebrafish offers a host of advantages for high-throughput small molecule screening, including translational appeal, high fecundity, and a unique set of developmental characteristics that support chemical treatment, genetic manipulation, and phenotype detection. High-quality genome sequencing and a complete annotation of zebrafish protein-coding genes have revealed that 71.4% of human genes have at least one zebrafish ortholog, and, reciprocally, that 69% of zebrafish genes have at least one human ortholog (Howe *et al.*, 2013). Notably, the conservation of functional domains, a category that includes drug-binding targets, nears 100% (Langheinrich, 2003). In a study that assayed 23 compounds known to cause QT prolongation in humans, 22 compounds tested positive in zebrafish, underscoring a high conservation of drug responses (Milan *et al.*, 2003). Similarly, toxic reactions in zebrafish, including teratogenic effects (Van Leeuwen *et al.*, 1990 and Yang *et al.*, 2009 and Selderslaghs *et al.*, 2009), are comparable to those in mammalian models (Parng *et al.*, 2002). Conservation of carcinogenic effects has also been supported (Spitsbergen *et al.*, 2000a and Spitsbergen *et al.*, 2000b). Taken together, genetic similarities between zebrafish and humans and comparable responses to chemical treatment between zebrafish and mammalian models provide a promising translation of newly identified therapeutic compounds into the clinic.

The simultaneous screening of thousands of small molecules requires a large number of viable, developmentally synchronized embryos. Females are typically capable of producing 100–300 embryos per week. Multiple matings through the setup of numerous breeding cages, therefore, can amass a sufficient number of embryos for large-scale screens. However, independent mating of breeding pairs can result in a final pool of mildly asynchronous embryos and requires a large amount of space and input of time. The invention of a specialized breeding vessel, the iSpawn (Figure 2), has effectively eliminated these obstacles (Adatto *et al.*, 2011). While maintaining a small footprint of 2.92 ft², the vessel provides a platform for the simultaneous mating of nearly 200 fish within a short window of time, resulting in spawning rates of up to 860 embryos per minute and a collection count of nearly

10,000 highly developmentally synchronous embryos (Adatto *et al.*, 2011). Embryo collection and equipment setup and breakdown are more efficient compared to conventional breeding methods, allowing large-scale screens to be completed in a period of weeks as opposed to months (Adatto *et al.*, 2011).

External development of embryos in an aquatic environment provides the critical advantage of screening small molecules *in vivo* and affords a variable of temporal control not previously possible with classical genetic mutagenesis studies. Small molecules are easily solubilized in water and absorbed through the skin and gills where, once internal, they are subject to metabolic modifications. Indeed, exposure of some compounds to an active metabolic pathway has proven vital for efficacy. Murphey and colleagues screened 16,320 small molecules in zebrafish for mitotic inhibitors and found 14 novel compounds with cell cycle activity that, despite numerous screens in mammalian cell lines using the same chemical library, were previously unidentified (Murphey *et al.*, 2006).

In addition to enabling metabolic activation of small molecules, *in vivo* screening using zebrafish affords other benefits over cell-based screening, without compromising the magnitude of assayed libraries. Use of the entire organism enables exposure of small molecules to all biological pathways, rather than to a subset of pathways specific to a single cell line. This advantage has been especially important in forward phenotype-based screens, where the pathways and genes targeted by a small molecule are initially unknown. Similarly, use of an intact organism to screen compounds allows for the expression of phenotypes resulting from the interplay of multiple pathways, cell-cell interactions, and cell-niche interactions.

The *ex utero* development of transparent embryos facilitate visual assessment of broad phenotypic changes during chemical screening. Early chemical screens utilized wild type (WT) embryos and visually scored abnormalities ranging from developmental delays to deformities in the central nervous and cardiovascular systems (Peterson *et al.*, 2000). Subsequently, the system was expanded to study animal behavior (Kokel *et al.*, 2010 and Kokel *et al.*, 2011), cardiac function (Milan *et al.*, 2003 and Peal *et al.*, 2010), and regeneration (Mathew *et al.*, 2007 and Oppendal *et al.*, 2010). To specify, the photomotor response (PMR) is a behavior-based phenotypic endpoint that measures animal activity in response to light stimulation. Assessing the PMR in 36 hpf zebrafish embryos exposed to a large library of chemical compounds revealed complex behavioral patterns and identified novel psychotropic drugs (Kokel *et al.*, 2010 and Kokel *et al.*, 2011). Another chemical screen performed in zebrafish identified bradycardia-inducing drugs, which later were shown to cause QT prolongation in humans (Milan *et al.*, 2003). Finally, a chemical screen of 2,000 bioactive compounds revealed 17 chemicals that inhibit fin regeneration in zebrafish embryos and elucidated key pathways involved in vertebrate tissue regeneration (Mathew *et al.*, 2007).

In recent years, chemical genetic screening has grown in complexity due to the characterization of mutant zebrafish lines, making it possible to identify chemical inhibitors of mutant phenotypes through chemical suppressor screens. Stern and colleagues—full study featured below—utilized *crash&burn* (*crb*), a cell cycle arrest mutant, to identify oncogenic

genes (Stern *et al.*, 2005). Chemical screening in a polycystic kidney disease mutant (*pkd2*) background identified inhibitory functions for histone deacetylase inhibitors trichostatin-A and valproic acid in kidney cyst formation (Cao *et al.*, 2009). Finally, chemical screening in *breakdance* (*kcnh2*) mutants revealed two compounds, flurandrenolide and 2-methoxy-N-(4-methylphenyl) benzamide (2-MMB), that rescue long QT cardiac phenotypes (Peal *et al.*, 2011).

The development of transgenic zebrafish lines has similarly expanded the scope of chemical genetic screens by incorporating readouts detectable by advanced microscopy. Transgenic manipulation can specifically label physiological structures, cellular lineages, and mature cell types. For example, the first chemical screen to identify anti-angiogenic compounds utilized the Tg (*VEGFR2:GRCFP*) fluorescent transgenic line (Tran *et al.*, 2007). Additionally, Tg(*fli:EGFP*) and Tg(*flk:EGFP*) are common transgenic lines that fluorescently label the complete vasculature, and have been used in chemical screens to identify modulators of cardiovascular development (Kitmbi *et al.*, 2009 and Le *et al.*, 2014) and anti-angiogenic compounds (Wang *et al.*, 2010). Another transgenic fluorescent reporter, Tg (*lyve1:egfp*)^{yz150}, labels lymphatic vessels through the expression of lymphatic vessel endothelial hyaluronan receptor 1, and has been used to identify FDA-approved drugs with anti-lymphatic activity (Astin *et al.*, 2014). Finally, transgenic lines may mark specific cell lineages or types. Tg(*lck:EGFP*), which labels T-cells, enabled the discovery of Lenaldekar as a novel and selective anti-leukemic compound (Ridges *et al.*, 2012). Future developments in zebrafish transgenesis, including the expansion of tissue specific lines and use of temporally controllable promoters, will contribute to the growing field of *in vivo* phenotype-based chemical genetics.

In vivo screening of molecules also provides important information on pharmacodynamics, pharmacokinetics, and toxicity, effectively combining drug discovery and animal testing. Bridging the gap between discovery and therapy has proven particularly fruitful through the screening and repurposing of FDA-approved drugs. For example, Gutierrez *et al.*—full study featured below—performed a screen in zebrafish to identify compounds amongst a library of FDA-approved drugs with activity against T-ALL. Perphenazine, an antipsychotic, was found to induce apoptosis in T-ALL cells through dephosphorylation of protein phosphatase 2A (PP2A) (Gutierrez *et al.*, 2014). Similarly, White and colleagues—also featured below—identified leflunomide, an FDA-approved anti-arthritis drug (Schiff *et al.*, 2000), as a novel melanoma therapeutic (2011).

An important advantage of the zebrafish model system that is manifested in many elements of high-throughput chemical screening is economy: of funds, time, and space. Zebrafish are relatively inexpensive to house and maintain compared to mammalian models, and only a small number of adult fish are required to produce thousands of embryos. Development of the iSpawn has reduced the amount of equipment needed to amass a large volume of embryos, as well as the input of time necessary for setup, collection, and breakdown (Adatto *et al.*, 2011). Small embryo size—approximately 1 mm in diameter—conveniently allows molecules to be screened in small, multi-well plate format, economizing on both space and the cost of chemical compounds. Finally, the marriage of drug discovery and toxicological

testing with use of an *in vivo* model is an efficient use of time and funds and provides a platform for rapid translation into the clinic.

2.3. Limitations and Future Directions of Small Molecule Screening in Zebrafish

The field of chemical genetics in zebrafish will continue to expand in the coming years due to the myriad of genetic and developmental characteristics that make the zebrafish system an excellent model for high-throughput chemical screening. While current technologies allow for a diversity of chemical screen designs, technological advancements in automated embryo handling and imaging will overcome current limitations and greatly expand the breadth and depth of the field.

Traditionally, the process of collecting, sorting, and arraying embryos into a multi-well format has been a time intensive task, representing a major bottleneck in high-throughput workflow. The introduction of robots that remove dead embryos, as well as selectively sort a specified number of embryos into multi-well plates has recently attenuated this obstacle. For example, the Complex Object Parametric Analyzer and Sorter, COPAS, (Union Biometrica, Holliston, MA) can selectively sort a desired number of embryos into individual wells, while simultaneously eliminating dead embryos. Another automated system, the ZebraFactor, is capable of filling a 96-well plate within 11 minutes (Graf *et al.*, 2011). Systems that both dechorionate and sort embryos are also available for use (Mandrell *et al.*, 2012), effectively reducing the burden of time placed on researchers. In addition to automated embryo sorting and arraying, more complex systems that integrate fluorescence-based technologies similar to fluorescent activated cell sorting (FACS) capabilities will be useful for future experiments (Union Biometrica).

Other limitations include constraints regarding chemical compounds. Screening of small molecules *in vivo* using zebrafish necessitates that compounds are readily soluble in water and easily absorbed by the skin and gills, thereby eliminating water insoluble chemicals as potential hits. For example, a study screening 23 drugs known to cause cardiotoxicity in humans resulted in 4 of 5 false negative results in zebrafish due to poor absorption. Drug efficacy of false negatives was confirmed by microinjection (Milan *et al.*, 2003). Thus, the hydrophilicity of compounds is an important consideration when designing a screen, and large-scale delivery of insoluble or impermeable compounds remains an obstacle. Further, compound permeability may be affected by the genetic background of the fish, and may vary from clutch to clutch. For this reason, it is beneficial to pool embryos prior to plate distribution.

The developmental stage of embryos can have a significant effect on the efficacy of a compound. For example, the epidermal layers of larvae and adults are less permeable to small molecules than the embryonic epidermal layer. Small molecules in larvae and adults are primarily taken up through the gills, rather than absorbed through the skin, and are thus subjected to different metabolic environments. Depending upon the developmental stage assayed, differences in drug distribution throughout the whole organism and in cell environment exposure can affect chemical activity and may lead to inconsistent results. Future chemical screens in adult zebrafish may require extensive characterization of pharmacodynamics and pharmacokinetics properties as a proof-of-principle.

Fluorescence imaging is a common endpoint for chemical screening, and the development of more powerful, automated, high-content imaging machines will provide a platform for improved embryo image acquisition. Fluorescent-based reporters rely on microscopes for signal detection, and a limitation includes the inability to visualize fluorescent expression in deep tissues. A benefit of high-powered confocal imaging is its ability to image deep tissues within the embryo. However, embryo orientation and positioning, time required for image acquisition, and the ability to uniformly analyze images remain challenges. Currently, researchers are developing microfluidics-based technologies, such as the Vertebrate Automated Screening Technology (VAST) Bioimager, that properly orient embryos such that imaging can be processed in a uniform, high-throughput manner (Pardo-Martin *et al.*, 2010 and and Wielhouwer *et al.*, 2011 and Chang *et al.*, 2012). The development of specific molds using 3-D printing technology can also aid in uniform positioning of embryos (Masselink *et al.*, 2014 and Wittbrodt *et al.*, 2014). Most importantly, the development of automated imaging methods will not only alleviate the burden of time that accompanies manual scoring, and but also improve upon the error rate attributed to human judgment.

Generally, high-throughput screening has remained challenging in juveniles and adults. The largest hurdle in using adult zebrafish in chemical studies is the difficulty of controlling drug delivery. Directly solubilizing chemicals into water for passive transport into the gills can be expensive and limits dosage control. Current techniques of drug administration in adults generally require anesthesia and include invasive approaches, such as retro-orbital injections (Pugach *et al.*, 2009), intraperitoneal injections (Kinkel *et al.*, 2010), and oral gavage (Collymore *et al.*, 2012). Therefore, current technologies render high-throughput and long-term drug administration a challenge in adult zebrafish.

The pigmentation of adult zebrafish also presents an obstacle, as phenotypes are less easily detected. The creation of a transparent adult zebrafish devoid of all pigment has addressed this issue to enable the visualization of internal phenotypes and organs (White *et al.*, 2008). The *casper* mutant, named for its 'ghost-like' transparent phenotype, has laid the groundwork for future studies, including suppression screens utilizing transgenics and fluorescently-labeled tumor cells to identify anti-cancer and anti-metastatic drugs. From an economic standpoint, maintenance of adult zebrafish requires more resources. Increased size of adults notably necessitates a greater space for drug delivery—compared to the compact multi-well plates used for embryos—and thus larger volumes of compound. Unlike early-stage embryos, adult fish must be anesthetized prior to visualization and manipulation. The creation of a high-throughput method for imaging up to 30 unanesthetized adult fish in multiple fluorescent channels, however, is alleviating this burden (Blackburn *et al.*, 2011).

Indeed, many researchers have opted to screen for embryonic markers, forgoing the availability of adult models, to circumvent the logistical obstacles associated with screening adults. However, a study in fin regeneration has revealed the feasibility of screening adults, albeit with a relatively limited number of compounds (Oppedal and Goldsmith, 2010). Similarly, Chen and colleagues performed a secondary transplant screen in adult zebrafish to identify compounds that inhibit embryonal rhabdomyosarcoma growth (Chen *et al.*, 2014). 12 hits from a large-scale screen that tested the effects of nearly 40,000 compounds on human embryonal rhabdomyosarcoma cell differentiation were combined with 83

compounds previously identified as having anti-RAS activity (Le *et al.*, 2013) for the adult screen (Chen *et al.*, 2014). The study indicates that adult screens may be more feasible as a follow-up to *in vitro* and *in vivo* embryonic screens performed on a larger scale.

3. Chemical Genetics and Cancer

3.1. Use of Chemical Genetics in Zebrafish to Identify Cancer Therapies

Cancer is a complex and heterogeneous disease that often involves the interaction of multiple signaling pathways. Chemical genetics offers an unbiased, broad-spectrum approach to identify molecules that interact with active oncogenic pathways, and to discover novel cancer therapies. The zebrafish is a valuable *in vivo* model for translational oncology (White *et al.*, 2013 and Yen *et al.*, 2014 and Barriuso *et al.*, 2015) due to its adaptability in transgenesis (Berghmans *et al.*, 2005 and Langenau *et al.*, 2005 and Langenau *et al.*, 2005), genome-editing (Foley *et al.*, 2009a and Foley *et al.*, 2009b and Sander *et al.*, 2011 and Hwang *et al.*, 2013 and Hwang *et al.*, 2013), transplantation (Langenau *et al.*, 2008 and Ignatius *et al.*, 2012), and imaging. Since the spontaneous development of cancer in the zebrafish is rare, transgenic cancer models are used to genetically and histopathologically mimic human cancers in the zebrafish. To date, a high-throughput screening approach has been used to identify targeted treatments for melanoma, leukemias (including AML and T-ALL), rhabdomyosarcoma, and prostate cancer (Table I). Small molecule screens have also been employed to find inhibitors of events related to cancer metastasis, including angiogenesis and lymphangiogenesis; cell cycle aberrations; and oncogenic pathways, including Ras, Hedgehog, and Wnt/b-catenin (Table I).

The reactivation of developmental programs that can accompany oncogenesis makes the zebrafish an attractive model for cancer studies. Embryos modeling cancer-initiating events have been used for high-throughput screens, economizing on time, space, and funds. For example, Le and colleagues ectopically expressed RAS during embryogenesis to investigate possible mechanisms at play in tumorigenesis. Using transgenic *hsp70-HRAS^{G12V}* embryos to screen for chemical suppressors of RAS signaling, two compounds were identified that subsequently had anti-tumor activity in both a zebrafish model of rhabdomyosarcoma and a human cell line of rhabdomyosarcoma (Le *et al.*, 2013). More simply, small molecules can be used to inhibit oncogenic pathways already active during development. Hao *et al.* used wild-type embryos to identify small molecule inhibitors of the Wnt pathway (Hao *et al.*, 2013). Hits were determined by a straightforward morphological phenotype, specifically dorsoventral patterning (Hao *et al.*, 2013). The screen identified a small molecule, Windorphen, that selectively killed human cancer cells with aberrant Wnt signaling, including colon and prostate cancer cells (Hao *et al.*, 2013).

The developing zebrafish embryo has been useful for identifying targeted therapies against cancer stem cells, a small population of quiescent, tumorigenic cells that contribute to metastasis and relapse. Screening *in vivo* avoids the difficulties of purifying these rare cells and culturing them *in vitro*. Using *Tg(hsp:AML1-ETO)* embryos, Yeh and colleagues screened a library of small molecules to identify novel compounds that inhibit the oncogenic effects of AML1-ETO (AE) on multipotent hematopoietic progenitor cells (Yeh *et al.*, 2009). Nimesulide, a selective COX-2 inhibitor, effectively antagonized AE activity (Yeh *et al.*,

2009) and was subsequently shown to inhibit the initiation and progression of xenograft tumors (Zhang *et al.*, 2013).

Here, we highlight three chemical genetic screens in zebrafish with an emphasis in elucidating cancer mechanisms and identifying novel cancer therapies. The first study features a chemical suppressor screening strategy to identify novel cell cycle molecules and pathways involved in cancer progression (Stern *et al.*, 2005). The second study illustrates the power and promise of repurposing FDA-approved drugs for cancer therapy with a rapid translation into the clinic (White *et al.*, 2011). Finally, the third highlights the value of genetic manipulation in the zebrafish model, through the use of inducible oncogenes and fluorescent reporters, in identifying novel T-ALL therapies (Gutierrez *et al.*, 2014).

3.2. Chemical Screen to Identify Genes Involved in Oncogenic Pathways

Identifying and understanding genes involved in cancer formation can aid in the development of targeted therapies. In 2005, Shepard and colleagues performed a zebrafish forward genetic screen to identify novel genes involved in cancer formation and identified the crash&burn (*crb*) mutant. This *crb* mutant exhibited signs of mitotic arrest due to an observable increase in mitotic and apoptotic cells and further molecular characterization of the *crb* phenotype identified a splice-donor mutation in the *bmyb* gene that resulted in decreased cyclin B1 expression, mitotic arrest, and genomic instability (Shepard *et al.*, 2005). In a follow-up investigation, Stern and colleagues employed a zebrafish-based chemical suppressor screening strategy to identify novel molecules that interact with the *bmyb* pathway. In 16 weeks, thousands of zebrafish embryos were spawned from heterozygote *crb* mutant parents, arrayed into a 384-well microplate, and treated with chemicals from the DIVERSetE library of 16,320 compounds (ChemBridge Corp).

To identify chemicals that interact with the *bmyb* pathway, the authors used whole embryo immunohistochemistry to assay for mitotic cells. A serine-10-phosphorylated histone 3 (pH3) antibody was used, as histone H3 is phosphorylated on serine 10 from late G2 to early M phase and dephosphorylated during anaphase. The chemical screen identified a single chemical that suppressed the *crb* mutant phenotype of mitotic arrest without having any mitotic effect in WT embryos. Stern and colleagues resynthesized the compound; validated the chemical structure and potency based on liquid chromatography, mass spectrometry, and nuclear magnetic resonance spectra; and named the chemical suppressor persynthamide. In Figure 3A, WT, heterozygote (Het), and mutant (Mut) *crb* embryos are stained for pH3 to quantify mitotic accumulation in the presence of the chemical hit persynthamide. The genotype was validated via restriction fragment length polymorphism. Figure 3B quantifies pH3-positive cells in the tails of 24 hpf embryos, and emphasizes the suppressive capacity of persynthamide, the chemical structure of which is depicted in Figure 3C. Treatment with persynthamide restored additional *crb* mutant phenotypes, such as deformed head morphology, cell death, and polyploidy.

This screen highlights the power of chemical screening in zebrafish. The rapid development of zebrafish embryos, coupled with high fecundity, enabled researchers to screen 16,320 chemicals within 16 weeks. The chemical suppressor screen performed in *crb* heterozygous mutants identified a novel chemical compound that interacts with the *bmyb* pathway. The

report demonstrates the advantages of using mutant zebrafish lines, chemical suppression of mutant phenotypes, and the elucidation of molecular characterization of cell-cycle mutants. The high degree of genetic conservation between zebrafish and humans, particularly the conservation of cell-cycle-related genes, allowed for a meaningful approach to study the mechanism of cell-cycle lethal mutant phenotypes.

3.3. Chemical Screen to Identify Melanoma Therapies

A major advantage of *in vivo* chemical screening is the ability to simultaneously assess drug efficacy and toxicity, and thus translate chemical hits with therapeutic promise more rapidly into the clinic. Using zebrafish embryos, White and colleagues performed a large-scale chemical screen and identified a novel function of an FDA-approved drug in the treatment of melanoma.

To better understand the events of melanoma initiation, White and colleagues investigated the potential interaction between the *BRAF(V600E)* activating oncogene, the most commonly mutated gene in human melanoma (Davies *et al.*, 2002), and developmental transcriptional programs. Transgenic zebrafish expressing human *BRAF(V600E)* driven by the melanocyte-specific promoter *mitf* develop tumors at an accelerated rate when crossed with *p53*^{-/-} mutants. Adult tumor cells aberrantly express the neural crest progenitor *crestin*, suggesting a return of tumorigenic melanocytes to a multipotent, neural crest progenitor state and implicating BRAF(V600E) in maintaining that state. It was hypothesized that events taking place in Tg (*mitf:BRAF(V600E)*);*p53*^{-/-} embryogenesis, namely the overlap between BRAF expression and active neural crest transcriptional programs, would mimic the initiating events of melanoma.

Based on the prediction that chemicals effective in suppressing neural crest progenitors would also be effective treatments for melanoma, a large-scale screen was performed using zebrafish embryos. 2,000 compounds were tested in wild-type embryos. After treatment, embryos were fixed for a secondary ISH assay to evaluate *crestin* expression. One compound of unknown function, NSC210627, strongly reduced *crestin* expression. Using the chemoinformatic approach, NSC210627 was found to have a similar structure to bequinar, a known dihydroorotate dehydrogenase (DHODH) inhibitor (McLean *et al.*, 2001), and inhibited DHODH activity *in vitro*. Leflunomide, another DHODH inhibitor and FDA-approved anti-arthritis drug (Schiff *et al.*, 2000), phenocopied NSC210627 *in vivo* and was used in future studies.

The effects of leflunomide were further characterized *in vivo* and *in vitro*. Embryos treated with leflunomide lacked melanocytes at 36–48 hpf, and exhibited a nearly complete lack of melanocyte progenitors at 24 hpf (Figure 4, A and B). Treatment of a human melanoma cell line with A771726, the active metabolite of leflunomide, decreased proliferation in a dose-dependent manner. Jointly treating melanoma cells with A771726 and the BRAF(V600E) inhibitor PLX4720 had a synergistic effect in suppressing proliferation. A similar effect was observed in a mouse xenograft model, with joint treatment resulting in nearly complete tumor regression in 40% of animals.

By selectively expressing oncogenic BRAF(V600E) in zebrafish melanocytes, White and colleagues were able to mimic melanoma initiation in embryos. Modeling melanoma onset in the zebrafish embryo provided a platform for a large-scale *in vivo* chemical screen aimed at identifying novel compounds that could suppress the neural crest. This study underscores zebrafish as a powerful model for drug discovery through large-scale *in vivo* chemical screening. By effectively combining drug discovery and animal testing and, further, by repurposing FDA-approved drugs, novel therapies can be rapidly translated into the clinic. Shortly after leflunomide was implicated in the suppression of melanoma, it jointly entered clinical trials with a BRAF(V600E) inhibitor.

3.4. Chemical Screen to Identify T-ALL Therapies

As in melanoma, a chemical genetic approach in zebrafish has recently proven fruitful in identifying novel T-ALL therapies through the repurposing of phenothiazines, a class of FDA-approved antipsychotics. Gutierrez and colleagues notably developed a fluorescence-based screen using inducible *rag2:dsRed2;rag2:MYC-ER;mitfa* transgenic zebrafish. The study highlights the advantage of genetic manipulability afforded by the zebrafish model system in large-scale chemical screening.

In an attempt to identify novel T-ALL therapies, Gutierrez and colleagues performed two screens in tandem: an *in vivo* screen in zebrafish and an *in vitro* screen in human T-ALL cells. Of note, a fluorescent reporter, *dsRed2*, was introduced into a previously established MYC-induced T-ALL zebrafish model (Langenau *et al.*, 2003 and Weng *et al.*, 2004 and Gutierrez *et al.*, 2013) to provide a straightforward platform for phenotype detection. Four libraries—including 4,880 FDA-approved drugs, drug-like small molecules, and natural products—were screened in *rag2:dsRed2;rag2:MYC-ER;mitfa* transgenic zebrafish larvae at 3 days post-fertilization (dpf). Larvae were simultaneously induced with 4-hydroxytamoxifen to activate the MYC-ER fusion protein. Chemicals were scored at 7 dpf for the ability to selectively kill MYC-overexpressing thymocytes, with either a complete loss of fluorescence or weak fluorescence constituting a hit (Figure 5A). Perphenazine, an FDA-approved phenothiazine antipsychotic, qualified as a chemical hit (Figure 5B) and was validated in a secondary screen by dose response.

Concurrently, the Broad Institute bioactives chemical library collection of 3,194 compounds—including 2,108 compounds from the *in vivo* screen—were assayed in KOPT-K1 cells, a NOTCH1-dependent T-ALL line. Compounds were evaluated for synergistic suppression of growth in combination with gamma-secretase inhibitors, which selectively target the NOTCH pathway. Perphenazine, again, scored as a top hit.

Follow-up studies revealed that perphenazine could suppress established tumors in zebrafish and T-ALL growth in culture in a Notch-independent manner by inducing mitochondrial apoptosis. Using ligand-affinity chromatography and mass spectrometry, protein phosphatase 2A (PP2A) was identified as a direct target of perphenazine. PP2A was proven to mediate the anti-leukemic effect of perphenazine through rapid dephosphorylation of substrates involved in oncogenic pathways, such as AKT, ERK, p70S6K, MYC, and BAD. Further, knockdown of PP2A subunits by shRNA was sufficient to impair the anti-leukemic effect of perphenazine.

The discovery of novel anti-leukemic activity by perphenazine that is Notch-independent holds great promise for the clinic, especially for patients in poor prognosis groups. While the high doses of perphenazine required to reach therapeutic benefit remain an obstacle in cancer treatment, the identification of PP2A as an important anti-leukemic agent will enable the development of new, targeted treatments. Notably, the ease of genetic manipulation in zebrafish—including the abilities to model T-ALL *in vivo* by selectively and conditionally over-expressing MYC in thymocytes, to introduce fluorescent reporters, and to assess relative fluorescent expression in a high-throughput manner—facilitated the identification of perphenazine amongst thousands of assayed compounds.

4. Conclusion

Chemical genetics is a powerful technique for identifying genes and biological pathways involved in development and disease, including cancer. The zebrafish system has been critical to the expansion of chemical genetics, as an economic, genetically manipulable model that is amenable to high-throughput, forward genetic, phenotype-driven screening. Importantly, *in vivo* large-scale chemical screening provides a platform for discovering novel therapies and translating them rapidly to the clinic. Future developments in automating large-scale high-throughput workflow, improving imaging technology for more sensitized phenotype detection, and expanding the current assemblage of zebrafish disease models will continue to advance the field.

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Figure 1.

The iSpawn is a specialized breeding tank designed to collect thousands of developmentally synchronous embryos. Approximately 200 adult zebrafish can breed in the shallow environment created by the mesh divider. Upwards of 10,000 embryos can be collected at the base of the vessel in minutes.

Figure 2.

Chemical screening workflow. (A) Developmentally synchronous embryos generated from adult zebrafish are collected and healthy embryos are arrayed into a multi-well format. (B) Chemicals at a specified dilution are added to the multi-well plate, and removed after the appropriate incubation period. (C) The embryos from individual wells are scored for the specified phenotypic endpoint.

Figure 3. Persynthamide suppresses mitotic accumulation in *crb* mutants. (A) Persynthamide abrogates mitotic accumulation in *crb* mutants as demonstrated by pH3 immunohistochemistry (B) Quantified levels of persynthamide-mediated chemical suppression. (C) Chemical structure of synthesized chemical, persynthamide.

Figure 4. Leflunomide suppresses neural crest development. (A) Leflunomide, a DHODH inhibitor, reduces *crestin* expression by *in situ* hybridization. (B) Leflunomide causes an absence of pigmented melanocytes.

Figure 5. Perphenazine has anti-T-ALL activity in vitro and in vivo. (A) The chemical screen design utilizes a 4-hydroxytamoxifen inducible fluorescent reporter in a transgenic zebrafish to visualize MYC-overexpressing thymocytes. (B) Perphenazine causes a decrease in MYC-overexpressing thymocytes in 7 dpf zebrafish larvae, as detected via fluorescence imaging.

Table I

Screening Cancer Phenotypes

Scoring Approach → Phenotype → Zebrafish → Reference

Reference	Phenotype
Astin <i>et al.</i>	Lymphangiogenesis
Chen <i>et al.</i>	Embryonal rhabdomyosarcoma
Gutierrez <i>et al.</i>	Leukemia (T-ALL)
Hao <i>et al.</i>	Wnt/b-catenin signaling
Le <i>et al.</i>	Rhabdomyosarcoma/RAS signaling
Murphey <i>et al.</i>	Cell cycle
Ridges <i>et al.</i>	Leukemia (T-ALL)
Stern <i>et al.</i>	Cell cycle
Tran <i>et al.</i>	Angiogenesis
Wang <i>et al.</i>	Prostate cancer/angiogenesis
White <i>et al.</i>	Melanoma
Yang <i>et al.</i>	Hedgehog signaling
Yeh <i>et al.</i>	Leukemia (AML)