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Review Article

The Zebrafish as a Tool to Cancer Drug Discovery

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Abstract

The ability of zebrafish to faithfully recapitulate a variety of human cancers provides a unique *in vivo* system for drug identification and validation. Zebrafish models of human cancer generated through methodologies such as transgenesis, gene inactivation, transplantation, and carcinogenic induction have proven similar to their human counterparts both molecularly and pathologically. Suppression of cancer-relevant phenotypes provides opportunities to both identify and evaluate efficacious compounds using embryonic and adult zebrafish. After relevant compounds are selected, preclinical evaluation in mammalian models can occur, delivering lead compounds to human trials swiftly and rapidly. The advantages of *in vivo* imaging, large progeny, and rapid development that the zebrafish provides make it an attractive model to promote novel cancer drug discovery and reduce the hurdles and cost of clinical trials. This review explores the current methodologies to model human cancers in zebrafish, and how these cancer models have aided in formation of novel therapeutic hypotheses.

Keywords: Zebrafish; Cancer; Drug discovery; Small molecule screens; Efficacy; Toxicity

Abbreviations

MPNST: Malignant Plural Nerve Sheath Tumor; T-ALL: T-cell acute lymphoblastic leukemia; hpf: Hours Post-Fertilization; DHODH: Dihydroorotate Dehydrogenase; B-ALL: B-cell Acute Lymphoblastic Leukemia; AML: Acute Myeloid Leukemia; MPN: Myeloproliferative Neoplasm

Current Challenges in Drug Discovery

The pharmaceutical industry is experiencing lapses in drug development productivity. The predominant drug discovery methodologies for the past 50 years have been target centric. The drug development process in its entirety, from compound identification through preclinical animal models, takes approximately 10-15 years [1]. A high number of compounds are often filtered out during the preclinical animal testing stage, due to failure to meet standards of Absorption, Distribution, Metabolism, and Excretion (ADME). This is because multiple iterations of *in vivo* studies are performed on preclinical animal models in later stages of drug development (i.e., prior to compounds being relinquished for human trials). Specifically, more than 70% of compounds in oncology fail in phase II clinical trials, while 59% of the remaining compounds are discarded in phase III due to intolerable toxicities [2]. To increase success rates, it is extremely important to test compounds using inexpensive, whole organism vertebrate models during early stages of drug development. Whole organism testing not only provides information on tissue specificity and toxicity, but also determines compound bioavailability that may not be accurately accounted for in a small number of murine models. The zebrafish has emerged as an ideal complementary model system for drug discovery, as it is capable of high throughput screening for discoveryof novel therapeutic compounds or testing of candidate cancer modulators. Research in the past few years has proven the potential of zebrafish to significantly improve the capacity of predicting clinical efficacy and reduce the time and money lost in pushing ineffective drugs to market [3].

The Advantage of the Zebrafish System in Drug Discovery

Zebrafish have emerged as powerful models for drug discovery and biosafety studies because they develop most of the organs found in mammals including those of the nervous, digestive, reproductive, immune, excretory, and cardiovascular systems [4,5]. Zebrafish have a number of unique advantages positioning them for rapid drug discovery and toxicity testing: (i) zebrafish generate large numbers of progeny, offering high confidence in statistical analysis; (ii) zebrafish can absorb compounds solubilized in water, making drug administration simple and feasible; (iii) zebrafish develop rapidly, allowing for assays of drug toxicities on organ development; (iv) the maintenance cost for zebrafish is less expensive than for mammals, decreasing the cost associated with animal husbandry [6]; (v) zebrafish and human share high molecular and genetic homologies, especially for enzymes and cell surface receptors [7]; (vi) zebrafish embryos are as accessible and proliferative as cell culture systems and thus lend themselves to being as applicable as *in vitro* systems; and (vii) multiple cancer models have been generated in zebrafish and proven similar to their human counterparts molecularly and pathologically, providing excellent tools for anti-cancer drug discovery through large-scale screens, candidate drug testing, and target identification [8,9]. Taken together, these features indicate that the zebrafish is a simple, cost-effective, and faithful model for both drug discovery and toxicological studies.

Zebrafish Models of Human Cancer

Zebrafish cancer models induced by chemicals

While maintaining zebrafish in laboratory conditions, researchers observed diseases developing in adult fish, including cancer. Later studies clarified that after exposure of certain mutagens, zebrafish spontaneously developed almost any tumor type known from humans with similar morphology and comparable signaling pathways. The

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Table 1: Zebrafish Cancer Models.

Methods for modeling zebrafish cancers	Chemicals or genetic alterations	Cancer types	References
Chemical Treatment	N-2-Flurenylacetamide	Liver	[13]
	N-nitrosodiethylamine	Cholangiolar tumors	[91]
	N-dimethylnitrosamine	Testicular germ cell tumor	[92]
	N-methyl-N′-nitro-N-nitrosoguanidine	Liver, Intestinal	[12]
	7,12-dimethylbenz[a]anthracene	Testis, Germ cell tumor, Vascular	[23]
Reverse genetics	*p53*	MPNST	[15]
	ptena	Ocular	[16]
	ptenb	Hemangiosarcoma	[16,17]
	apc	Liver, intestinal	[18]
	nf1a	Gliomas	[19]
	nf1b		
Forward genetics	Ribosomal protein gene	MPNST	[21]
	separase	Epithelial tumors	[23]
	bmyb	Liver, Intestinal	[24]
	Genomic instability mutations	MPNST/others	[22]
		Testicular germ cell tumor, Vascular tumor	[12]
Transgenesis	*TEL-AML1*	B-ALL	[27]
	Myc	T-ALL	[28-30]
	MYC	T-ALL	[31]
	Akt2	T-ALL	[31]
	NOTCH1	T-ALL	[32]
	MY5T3-NCOA2	AML	[33]
	NUP98-HOXA9	MPN	[44]
	KRASG12D	Rhabdomyosarcoma	[34,35]
	BRAF-V600E	Melanoma	[36]
	HRASG12V	Melanoma, liver cancer	[37,38]
	HRASG12V	Melanoma	[39]
	MYCN	Neuroendocrine tumor, Neuroblastoma, MPN	[40-42]
	Xmrk, kras, Myc	Liver cancer	[43]

most common locations for this spontaneous neoplasia to arise include gut, thyroid, and liver. Lower levels of spontaneous neoplasia occur in blood vessels, brains, and gills. In light of spontaneous tumor acquisition, detailed chemical approaches to induce cancer have been developed [10]. To chemically induce cancer, zebrafish are soaked in water dissolved with carcinogens for varied periods of time. Advantageously, zebrafish can endure treatments at a variety of chemical concentrations and durations. For instance, smaller doses, from 5 mM or less can be applied for up to 24 hours, while doses greater than 20 mM are to be applied for 8 hours or less [11]. The treatment of zebrafish with the mutagen 7,12-dimethylbenz(a) anthracene induces the broadest range of tumors, from epithelial tumors in intestines to mesenchymal tumors in blood vessels and lymphoid malignancies (Table 1) [12]. Treatment with N-nitrosodiethylamine is reported to induce pancreatic and liver carcinomas, while N-nitrosodimethylamine specifically induces liver tumors (Table 1) [13].

Zebrafish cancer models resulted from tumor suppressor inactivation

Reverse genetics aims to discover the function of a gene by characterizing phenotypic changes upon gene inactivation. Targeting, and subsequent inactivation of specific tumor suppressor genes has led to zebrafish cancer models of Malignant Plural Nerve Sheath Tumor (MPNST), ocular, liver, and intestinal cancer [14,15]. These cancer types are modeled through silencing of the p53, ptenb, apc, or nf1tumor suppressor genes respectively (Table 1) [15-19]. Targeted in activation of tumor suppressor genesis done by taking advantage of: site-directed mutagenesis, recombination mediated by Cre or Flp recombinases, Targeted Induced Local Lesions In Genomes (TILLING), Zinc Finger Nucleases (ZFN), Transcription Activator-Like Effector Nucleases (TALENs), or Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated (CRISPR/Cas) technologies [12].

In forward genetic screens, mutations are introduced to the adult zebrafish's genome through chemical, viral, or transposon-based mutagenesis. The progeny of these mutagenized adult zebrafish are screened for abnormal phenotypes. Genes that harbor genetic mutations are then identified through gene mapping, sequence analysis, and phenotype validation. Using zebrafish in forward genetic screens provides a powerful approach to identify cancer susceptible or novel modifier genes in a specific oncogenic cascade based on their ability to accelerate or suppress tumor phenotypes [20].It has been found that mutations in genes encoding ribosomal proteins have led to development of MPNST, as well as mutations affecting genomic instability that have also been associatedwith MPNST in zebrafish [21,22]. Shephard et al. have discovered that the *separase* and *bmyb*loss-of-function mutants are susceptible to liver and testicular cancers respectively, through forward genetic screens (Table 1) [23,24].

Transgenic zebrafish cancer models

Transgenic zebrafish expressing mammalian oncogenes provide a convenient platform for modeling human cancers through the misexpression of wild-type or constitutively active form of oncogenes under a zebrafish tissue-specific promoter. To generate transgenic zebrafish models, exogenous DNA is microinjected into one-cell-stage zebrafish embryos. Traditionally, linear or circular DNA plasmids, or artificial bacterial chromosomes are injected into fertilized zebrafish eggs. A large number of eggs need to be injected and screened to compensate for low germline transmission of the transgene of interest to the F1 generation. As transgenic lines are passed on through generations, repetitive DNA becomes susceptible to methylation,

Table 2: Small Molecule Screens with Zebrafish Cancer Models.

Cancer Type	Zebrafish Model	Compound Library Name	Small Molecules Identified	References
Leukemia	*lck:EGFP*	ChemBridge DIVERSet	Lenaldekar (LDK)	[73]
T-ALL	*rag2:MYC-ER;mitf1a:mitf1a; rag2:dsRed2*	Prestwick Chemical Library Spectrum Collection	Phenothiazine	[74]
T-ALL	*rag2:EGFP-mMyc;CG2*	Compounds obtained from Sigma Aldrich	Vincristine and Cyclophosphamide	[75]
AML	Xenotransplantation of K562, K562-R, JURKAT, NB4 human leukemia cell lines into wild-type zebrafish	Imatinib, all-trans retinoic, mafosamide, cyclopjosphamide, 4EGI-1	Imatinib	[76]
ALDH+ myelogenous leukemia	Xenotransplantation of ALDH+ and ALDH- leukemia cells into *fli1:EGFP;Casper*	imatinib, dasatinib, parthenolide, TDZD-8, arsenic trioxide, niclosamide, salinomycin, and thioridazine	Imatinib	[77]
AML	*Hsp70:AML1-ETO*	SPECTRUM library Microsource Discovery Systems	Nimesulide	[48]
Melanoma	*mitfa:BRAF(V600E); p53 -/-*	SPECTRUM library Microsource Discovery Systems	Leflunomide	[46]
Pancreatic Cancer	Morpholinos and chemical screens	selective RAR antagonists	RAR antagonist Ro-41-5253	[93]
Human Carcinoma	Xenotransplantation of YD10B and HSC-2 human oral squamous carcinoma cell lines; DLD-1 and HCT116 human colorectal carcinoma cell lines into wild-type zebrafish	Tagged triazine molecules	BII-B9 Paclitaxel	[94]

leading to the silencing of transgenes [4,5]. Modifications of these early transgenic techniques have led to the development of transposon- or I-SceI meganuclease-mediated transgenesis approaches that significantly improved germline transmission rates in zebrafish [25,26].With these improved techniques, modeling human cancers in zebrafish through transgenesis becomes much easier. There are multiple types of cancers in zebrafish developed through the use of transgenesis (Table 1) [27-44]. MYC-induced T-cell Acute Lymphoblastic Leukemia (T-ALL) and melanoma models in particular have not only been used extensively to gain mechanistic insights into disease pathogenesis, but also in small molecule screens to successfully identify candidate therapeutics for human cancers (Table 2) [25,29,30,32-37,39,40,45-48].

Zebrafish xenograft models of human cancer

An additional methodology to establish cancer models involves the transplantation of human cancer cells into zebrafish embryos. Zebrafish lack an innate immune system until 72 hours post-fertilization (hpf) and a mature adaptive immune response until 4 weeks of life [49]. Therefore, human cancer cell lines, purified subpopulations of cancer cells or primary patient cells can be directly injected into zebrafish embryos to study many aspects of tumor biology, such as vasculature remodeling, cancer invasion, and metastasis [50,51]. So far, multiple types of human cancer cell lines and primary patient samples, including gastrointestinal, neuroendocrine, leukemic, and melanoma clinical tumor samples, have been successfully transplanted into 48-hpf zebrafish embryos and demonstrated their usefulness in studying cancer pathogenesis as well as novel drug screening and therapeutic testing of candidate cancer drugs. Invasiveness and micrometastasis of primary human tumors occurs within 24 hours of transplantation [52]. These zebrafish xenograft models of human cancer are especially useful in drug screens allowing for the simultaneous examination of *in vivo* efficacy and toxicity of candidate drugs [53]. Finally, the advent of pigmentless *Casper* adult fish has enabled visualization of tumor cell proliferation and dissemination in transplanted recipients beyond zebrafish embryonic stages. Adult zebrafish have three distinct classes of pigment cells: black melanophores, reflective iridophores, and yellow xanothophores. *Nacre* mutant zebrafish lack melanocytes, while *roy orbison* zebrafish lack iridophores. *Casper* zebrafish are double mutant for *nacre* and *roy* lacking both melanocytoes and iridophores throughout embryogenesis and adulthood. *Casper* permits all organs to be seen with stereomicroscopy [40].

Basic Design of Small Molecule Screens using Zebrafish

The ultimate goal of small molecule library screens utilizing zebrafish is to record the greatest number of small molecules that can modulate the activity of zebrafish proteins. Thus, the best molecular library should include as much chemical diversity as possible with a variety of core chemical structures. Moreover, it is of best interest to develop a chemical library that has a high percentage of compounds with similar and consistent physiochemical properties. Chemical treatments can occur at any point during development allowing study of gene inactivation effects by small molecule inhibitors throughout fish development. Moreover, the chemical dosage can be controlled which is advantageous when studying essential functions of tissue specificity [54,55].

The most common chemical library applied in zebrafish screens is the DIVERSet E from Chembridge. This chemical library contains 50,000 compounds occupying a broad pharmacophore space, while excluding non-drug like compounds with undesirable chemical groups and structures. Using the DIVERSet E, Peterson et al., Sternson et al., and Murphey et al. have evaluated phenotypic changes in the central nervous system, cardiovascular system, pigmentation, and organogenesis respectively [56-58]. Phenotypic changes in hematopoiesis are evaluated after application of the NINDS custom collection from the NIH/NINDS, the Spectrum collection from Microsource, and the ICCB collection of known bioactive compounds from BioMol [59]. Changes in embryogenesis and angiogenesis are evaluated through application of 1,3-dioxane library from the Schreiber lab, trisubstituted triazines from the Chang lab, and the LOPAC1280 chemical library from Sigma Aldrich [60-62].

In general, drug screens can be divided into target- or phenotype-based approaches [54,63]. In target-based approaches, the aim is to

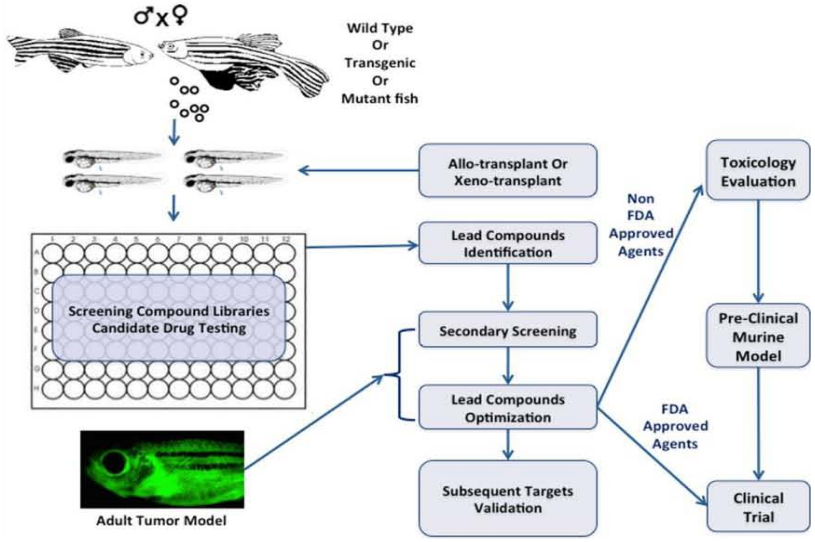

Figure 1: Integrating zebrafish into the cancer drug discovery platform. Zebrafish embryos from wild-type, transgenic, or mutant fish are arrayed into a plate with candidate agents or compound library to screen for small molecules that elicit desired phenotypes in embryos. Prior to drug treatment, embryos can also be transplanted with either zebrafish or human cancer cells to determine a compound's effect in killing tumor cells or inhibiting their migration properties. Adult zebrafish with cancer can be treated with identified or modified compounds to validate and prioritize the lead compounds for preclinical and clinical testing. Anti-cancer agents of unknown biological properties must undergo a toxicological and pre-clinical evaluation prior to clinical tests, while FDA-approved drugs can directly enter human clinical trials.

discover novel probes for defined biological pathways or molecular targets. When this approach is imparted in screening, a greater quality of hits can be generated instead of quantity. A well-designed screen should be able to inform not only the activity of the compounds directed against a particular target, but also other properties of the compounds, such as their permeability, specificity, and off-target effects [55]. Furthermore, target-based chemical screens can either be engineered to report the activity of a specific pathway or they can be formatted to identify compounds that phenocopy a known pathway disruption. Although target-based approaches can lead to the discovery of compounds directed against a specific protein or its related pathways, compounds identified in this manner may not significantly modify the disease of interest [64].

Phenotype-based approaches possess unique advantages in discovering compounds that either elicit a disease phenotype or conversely, rescue a disease phenotype. Novel or well-characterized pharmacological probes are screened in order to identify compounds that induce or modulate a specific phenotype. In their simplest forms, phenotype-based screens might involve searching for an agent that suppresses a cell cycle defect in embryos or rescues mutant phenotype affecting cancer genes. Screens for reversal or prevention of disease phenotypes in an organism are powerful because it allows for the identification of promising chemotherapeutics without bias about which targets are involved in disease pathogenesis. Moreover, many potential targets can be assessed in one screen, and bioactive compounds identified would be of high quality given that the screen output is a suppressor of a disease phenotype in a whole organism [65].

Throughput assays are another basic design of small molecule screens that consist of a small lot of well-characterized pharmacological compounds with known bioactivities. This enables generation of biological connections and hypotheses to be tested quickly since background information about the relevant subset of pharmacological compounds is available [66]. Finally, novel modifier screens can be employed to either identify or rescue disease-related phenotypes. These drug screens involve large libraries of uncharacterized compounds followed by significant efforts to ascertain the mechanisms of action of the identified hits in order to pursue clinical testing [67,68].

Application of Zebrafish in Cancer Drug Discovery

Suitability of zebrafish embryos for anti-cancer drug screens

A remarkable range of biological and disease processes can be studied in the early stages of zebrafish development, but may be limited when using adult zebrafish. Importantly, researchers made the connection between abnormal embryonic phenotypes and increased cancer susceptibility in adulthood, allowing chemical screens to be performed during embryogenesis in order to identify potential compounds and targets that could influence carcinogenesis. To rapidly estimate the therapeutic efficacy of small molecule compounds, wild-type, transgenic, or mutant zebrafish embryos are incubated with the compounds of interest in water (Figure 1). Because the embryos are optically transparent, it is possible to detect functional and morphological changes in internal organs without having to kill and dissect the organism post-compound treatment, a significant advantage over the use of other vertebrate models. Quantitative analysis using microplates can also facilitate comparisons between zebrafish embryos incubated with compounds and vehicles. Microplate analysis of embryos is similar to cell based assays, and is used for primary compound screening at high throughput cores. The small size of zebrafish embryos enables them to be arrayed in a variety of plate formats (12, 24, or 96-wells) and bathed in water containing the compound of interest (Figure 1) [65].

The use of multiple transgenic lines with fluorescent reporter molecules further enables detailed analysis of functional and morphological changes [69]. For instance, when compounds are fluorescently-labeled, it facilitates direct visualization of absorption into transparent embryos. Compound excretion following treatment can be observed and measured based on fluorescence properties as well [40]. In the early 2000s, forward chemical screens using zebrafish embryos led to the identification of compounds affecting vertebrate development. These embryo screens narrowed down the number of molecules for future testing in adult zebrafish models of tumor and mammals (Figure 1) [51]. As multiple zebrafish xenograft models of cancer are established, researchers have demonstrated their efficacy in small molecule screens. This is accomplished by soaking xenografted embryos in a compound solution or treating cancer cells with small molecules before implantation into transparent embryos (Figure 1) [45].

Fortunately, many toxic responses have been conserved between zebrafish and human toxicological studies.Therefore, zebrafish embryos are valuable in the assessment of system toxicity, which can be evaluated using overall embryonic mortality as a metric from which the working range of a compound can be determined. To do this, embryos are arrayed in a multi-well plate and exposed to drugs with a dose gradient to determine the maximum tolerable dose. Preliminary hits from screening can all be tested for toxicity as a means of prioritizing compounds [70,71].

Adult zebrafish for cancer drug validation

Adult zebrafish are often limited in their capacity to aid in drug screenings as adult zebrafish are relatively larger and less transparent. With the availability of multiple zebrafish models of cancer (Table 1), investigators can treat adult fish with tumors to validate the antineoplastic efficacy of candidate agents. Dissemination of tumors throughout the zebrafish can occur at varying rates, typically from 5 to 10 days post transplantation. Moreover, the appearance of these disseminated tumor cells may not occur until 2-3 weeks post transplantation. The innovation of the transparent *Casper* zebrafish has improved opacity in adult zebrafish [72], and provided a tool for exploration of tumor dissemination mechanism and subsequent metastatic growth throughout fish development. Advantages of using the *Casper* fish, in combination with syngeneic transplantation, include *in vivo* monitoring of tumor engraftment and migration following transplantation.

Advances using zebrafish in cancer drug discovery

Collectively, the use of embryo screens and drug validation in adult zebrafish models of cancer represents the ideal means to identify and prioritize candidate agents for further testing in mammals (Figure 1). Researchers have taken advantage of the zebrafish cancer paradigm and have successfully identified candidate compounds for cancer treatment. A novel high-content *in vivo* screen was conducted by Ridges et al. using genetically engineered T-cell reporting zebrafish larvae (*lck:EGFP*) (Table 2) [68]. By exploiting the developmental similarities between immature and malignant T-cells, the activity of small molecule libraries was assayed against immature T-cells with a corresponding visual readout in zebrafish larvae. It was found that the compound, Lehaldekar, was able to abolish immature T-cells in developing zebrafish without affecting cell cycle in other cell variants. Moreover, Lehaldekar was tolerated in murine models. Notably, Myc-driven T-ALL in adult zebrafish was induced into remission upon Lehaldekar treatment [73].

Gutierrez et al. designed a fluorescence-based small molecule screen to identify compounds that were selectively cytotoxic to MYC-overexpressing thymocytes in a MYC-induced (activated by tamoxifen) T-ALL background (Table 2) [74]. At three days post fertilization, FDA-approved drugs were added to embryos. Four days later, dsRed2 fluorescence expression in thymocytes was imaged via microscopy. Screening for the decreased dsRed2 expression in thymocytes lead to the identification of perphenazine, an antipsychotic drug, with antileukemic activity. Importantly, perphenazine was also found as an antileukemic agent in a parallel cell-based screen, and validated to induce apoptosis in fish, mouse and human T-ALL cells [74].

Mizgrev et al. aimed to validate the efficacy and faithfulness of zebrafish embryos in drug discovery. T-ALL was induced in zebrafish by mosaic expression of EGFP-fused *mMYC* transgene under a lymphocyte-specific promoter rag2 (Table 2) [75]. Subsequently, primary tumor cells were transplanted into recipient zebrafish larvae that were treated with vincristine and cyclophosphamide five days post leukemia engraftment. Drug efficacy and relevance toembryonic development was determined based on a compound's ability to increase larvae lifespan [75].

Pruvot et al. injected human leukemia cell lines or blasts from patients with acute myelogenous leukemia into zebrafish embryos at 48 hpf (Table 2). Compounds that demonstrated no toxicity in normal zebrafish embryos and decreased leukemia burdens in xenografted zebrafish were ideal, ultimately validating anti-leukemic efficacy of imatinib and oxaphorines [76]. Similarly, Zhang et al. injected purified human leukemic stem cells into zebrafish embryos based on kusabira-orange fluorescence [77]. Post-transplanted fish were treated with selected therapeutic agents (e.g. imatinib, dasatinib, parthenolide, etc.). Cell proliferation and migration were subsequently evaluated using high-content imaging, and recapitulated the ability of the drugs' to inhibit LSCs in both *in vitro* and murine studies – thus validating their methodology for future anti-LSC drug discovery (Table 2) [77].

Yeh et al. created an assay with zebrafish embryos that faithfully recapitulated the effects of the oncogene AML1-ETO to block cell differentiation in multipotent progenitor cells (Tables 1 and 2). A chemical screen of 200 bioactive chemicals was then performed in an effort to find a compound that would suppress the differentiation blockade of oncogenic AML1-ETO on hematopoietic progenitor cells. The Cox2 inhibitor nimesulide was identified as the lead hit of the screen. Their follow-up study demonstrated a previously unknown role for COX-2 and β-catenin in AML1-ETO-mediated hematopoietic differentiation [48].

Recently, White et al. preformed a small molecule screen that identified an inhibitor of Dihydroorotate Dehydrogenase (DHODH), leflunomide, which diminished the self-renewal of mammalian neural crest stem cells (Table 2) [46]. This chemical screen was performed using transgenic mitfa:*BRAF*(*V600E*) zebrafish embryos that had defective p53 activity. The inhibition of DHODH through leflunomide alone or in combination with an oncogenic inhibitor of

BRAF (V600E) was able to successfully suppress melanoma growth *in vitro* and in murine models [46].

The Zebrafish as a Model for Studying Tumor Metastasis

Tumor metastasis is an extremely complex, multistep cascade [78]. In total, there are at least four major steps involved: the local invasion and intravasation; the survival of tumor cells in circulation; the extravasation (the exit of the tumor cells from vasculature); and the distant proliferation and colonization [79]. By coupling optical transparency with the differential fluorescent labeling of tumor cells, inflammatory cells, and vasculature, zebrafish enable noninvasive, real-time visualization of metastatic events *in vivo*, distinguishing among each of the cell types and their dynamic interactions leading to intravasation and metastasis [80]. In particular, zebrafish xenograft models of cancer faithfully recapitulate the metastatic potentials of human cancer cells as in xenografted mouse models and in patients [81,82]. As such the zebrafish is particularly well suited for real-time monitoring of metastatic events *in vivo* [80]. The generation of *fli1:EGFP* and *flk1:mCherry* fish, in which the vasculature expresses EGFP or mCherry fluorescent protein, provides unprecedented high-resolution imaging of complex intravasation and extravasation processes [83,84].

Stoletov et al. combined the use of *fli1:EGFP* transgenic fish and fluorescently-labeled human cancer cellsin high-resolution confocal microscopy studies, demonstrating the role of RhoC and VEGF in intravasation [85]. Using a similar methodology, they subsequently visualized extravasation dynamics of metastatic tumor cells in zebrafish [86]. Their findings show that different from intravasation, extravasation does not require damaged vessels or vascular leakage, but instead is induced by local vessel remodeling (i.e., clustering of endothelial cells and cell-cell junctions). The availability of transparent *Casper* fish makes it feasible to study tumor cell intravasation and metastasis in adult fish. Using *Casper; fli1:EGFP* fish, Feng et al. demonstrated that elevated S1P1 levels inhibit lymphoma cell intravasation, whereas AKT activation or an S1P1 antagonist can overcome this blockade [87]. Ignatius et al. showed that in embryonal rhabdomyosarcoma (ERMS), myf5+ ERMS-propagating cells do not intravasate, while the more differentiated myogenin+ ERMS cells can readily invade the vasculature [88]. The ability of studying metastatic events in zebrafish coupled with the high fecundity of zebrafish suit this organism for small molecule screens to identify novel antimetastatic agents, especially repurposing the FDA-approved drugs.

The Future Perspectives

Due to their genetic similarities to humans, the zebrafish serve as a relevant *in vivo* system to complement mammalian systems. The optical clarity of zebrafish embryos and mutant *Casper* adult zebrafish has provided a foundation for assessment and imaging of tumor phenotypes through application of novel or known compounds. The embryonic zebrafish serves as the jumping off point for lead compound identification, while validation in adult zebrafish allows for longitudinal observation of compound impact on tumor cell growth, dissemination, and metastasis in an anatomically relevant system. Additionally, zebrafish allow for the assessment of absorption, distribution, metabolism, excretion, and toxicology properties of thousands of chemical compounds. These assessments are done at much quicker speeds, lower costs, and greater scale and efficiency. Although only being applied in small molecule screens for a few years, zebrafish have already demonstrated their great potential in cancer drug screens and novel compound identification.

The establishment of zebrafish cancer cell lines, especially for the ones with known and relevant oncogenes,would be advantageous for functional cell-based studies and would also enable the pre-screening genetic and chemical modifiers of cancer. Without a doubt, anti-cancer screens in zebrafish will be rapidly improved as robots and more readout automation are implemented in the procedures. An automated micro-injector allows a greater number of fish to be xenografted with high accuracy, improving the standardization of the screens and allow a large-scale screen to be performed in a shorter time frame [89]. Furthermore, automated imaging system (or an ImageJ plugin) optimized to detect or quantify a specific phenotypic feature of interest will drastically improve the readout quality and lower extensive labor necessary to perform such screens and identify candidates molecules [90]. With automation and standardization of these procedures, zebrafish can contribute to personalized medicine. For instance, *ex vivo* screening of cancer cells from an individual patient's could be conducted in a zebrafish xenograft model for the testing of multiple drugs and drug combinations, and the readout would inform clinicians on measures of both drug potency and toxicity, improving therapy selection on a case-by-case basis.

While we continue to maximize the use of zebrafish in high-throughput screens, we must keep in mind that the anatomical and molecular differences of zebrafish with humans may cause the elimination of a fraction of the hits generated. Thus, the human relevancy of compounds identified through the use of the zebrafish models must be carefully investigated. Because it still remains unclear to what degree the molecules discovered in zebrafish screens impart similar effects in humans, the predictive toxicity in zebrafish may not equate to that of human toxicity. Fortunately, so far, tested compounds (e.g., ones in regards to cell cycle affecters) showed a 50-70% similarity in effect between mammalian and zebrafish cells [3]. An even greater degree of drug conservation of 95% was observed in treating zebrafish and human with cardiac modulators [4]. The high degree of conservation between fish and man further establishes the usefulness of the zebrafish as an important tool to human cancer drug discovery.

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