Review

Cancer Research

Zebrafish: Speeding Up the Cancer Drug Discovery Process

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Abstract

Zebrafish (*Danio rerio*) is an ideal *in vivo* model to study a wide variety of human cancer types. In this review, we provide a comprehensive overview of zebrafish in the cancer drug discovery process, from (i) approaches to induce malignant tumors, (ii) techniques to monitor cancer progression, and (iii) strategies for compound administration to (iv) a compilation of the 355 existing case studies showing the impact of zebrafish models on cancer drug discovery, which cover a broad scope of scenarios. Finally, based on the current state-of-the-art analysis, this review presents some highlights about future directions using zebrafish in cancer drug discovery and the potential of this model as a prognostic tool in prospective clinical studies. *Cancer Res; 78(21); 6048–58. ©2018 AACR.*

Introduction

Cancer is a worldwide disease, being one of the main causes of morbidity and mortality at present. According to the World Health Organization, there will be an increase of 18.1 million new cancer cases and 9.6 million cancer deaths (1).

Traditionally, the murine model has been used in research as an *in vivo* model organism. However, *Danio rerio*, also known as the zebrafish, owing to its small size, heavy brood, and rapid maturation time, has emerged as an important new cancer model that complements what can traditionally be achieved in mice and cell culture systems. A wide range of assays can be carried out in this model, from target discovery, target validation, or toxicological studies to the generation of tumors to perform the corresponding *in vivo* efficacy tests (e.g., screening molecules; refs. 2, 3).

The zebrafish model possesses unique advantages that establish it as a versatile tool in research. (i) Zebrafish generates large numbers of progeny, offering high confidence in statistical analysis (4). (ii) Human and zebrafish share a high grade of similarity: 71% of human proteins and 82% of disease-causing human proteins have an ortholog in zebrafish (5). (iii) Husbandry expenses are reduced compared with mammals, owing to the inexpensive maintenance that they require (4). (iv) Zebrafish absorbs molecules that are dissolved in water, allowing feasible drug administration (6). (v) Some processes can be directly observed in the living animal due to the transparency of zebrafish embryos and the recent development of the *casper* zebrafish line, which is deficient in pigments (7). (vi) Many zebrafish disease models have been described so far due to the development of transgenic and mutant lines (8).

Zebrafish as a Model Organism in Cancer Research

In terms of cancer research, the zebrafish model has advantages against traditional cell culture assays, as a broader range of phenotypes can be tested (9). Zebrafish and mammals share common molecular pathways of tumor progression (10). Likewise, more than 130 distinct genes in zebrafish liver tumors present similar expression to liver human cancer profiles, correlating with histologic tumor type, grade, and stage. In fact, Zheng and colleagues proved that transgenic zebrafish models share molecular signatures with human hepatocellular carcinoma (11).

There are several approaches to generate human cancer in zebrafish, such as development of mutant and transgenic lines and transplantation of tumor cells (Fig. 1A). Each methodology has several advantages and disadvantages, which are described in Supplementary Table S1. The selection of the zebrafish stage in which experimentation should be carried out depends on the aim of the study, as each developmental stage presents some benefits (Supplementary Table S2). Embryos are most commonly used when the main purpose of the study is the visualization of a concrete tumor process, as their bodies are transparent and allow for microscopy observation. In addition, cancer develops more rapidly in embryos, showing tumor formation by 2 days after the induction. Consequently, they could be employed in projects that demand rapidity, such as imaging cancer processes or screening campaigns. By contrast, adults offer a more realistic *in vivo* model, as all of their organs and immune systems are developed; however, cancer establishment requires from 10–14 days to 1 month (12).

Mutant lines

The mutated cancer driver genes usually dominate cancer proceedings and determine the future of tumorigenesis (13). However, cancer initiation processes cannot be observed, and some operable and time-saving approaches are necessary to manipulate the zebrafish genome and to mimic cancer

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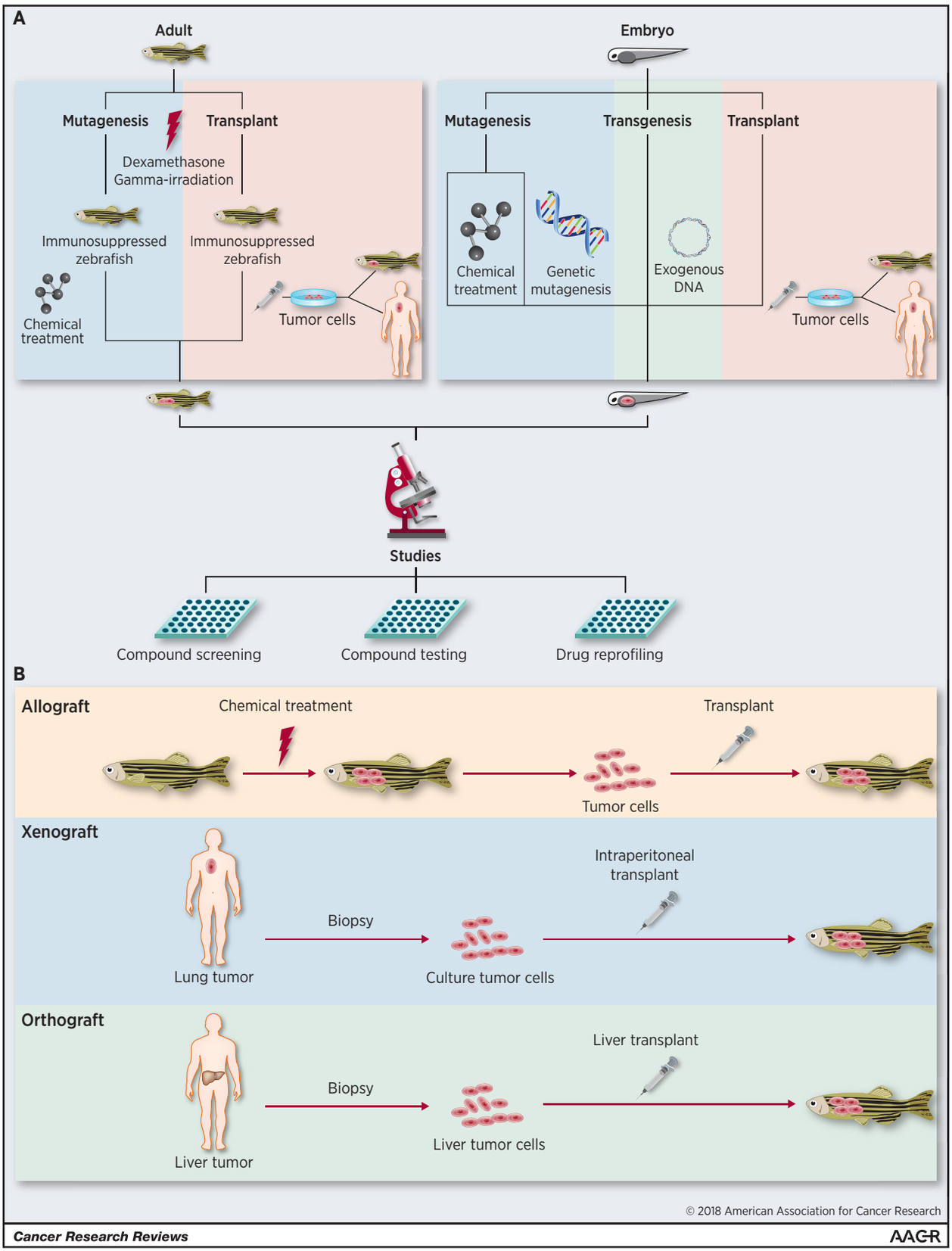

Figure 1.
A, Methods of cancer generation in adult and embryo zebrafish. **B,** Transplant assays in zebrafish.

initiation and progression. There are different ways to induce cancer in zebrafish, such as chemical mutagenesis, irradiation mutagenesis, insertional mutagenesis, which can be transposon-based, or viral vector mutagenesis. Until now, researchers forced the development of several cancer types using chemicals by adding carcinogens to the water, such as dibenzo(a,l)pyrene (DBP), 7,2-dimethylbenz(a)anthracene (DMBA), N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), N-dimethylnitrosamine (DEN), N-nitrosodiethylamine (NDMA), and N-ethyl-N-nitrosourea (ENU; Supplementary Table S3).

The genome engineering field has experienced an unprecedented rate of growth in recent years since the introduction of designer endonucleases. Genome engineering has not gone far in the field of zebrafish, and researchers use reverse genetics to induce cancer in zebrafish. These methods could imply the inactivation of a concrete gene or the conditional gene regulation through genome editing. The most used techniques to edit the genome and its application in zebrafish is shown in Supplementary Table S4. Other techniques to perform reverse genetic alterations such as morpholinos and RNAi have been less studied due to the limitations that are present (14, 15).

Transgenic lines

Researchers induce transgenic zebrafish models by microinjecting exogenous DNA into one-cell-stage zebrafish embryos, which originate misexpression of wild-type or constitutively active from oncogenes under a zebrafish tissue-specific promoter (16). The major drawback of this method is the difficulty in generating stable lines, as the deleterious effects that strong oncogenes could cause. Thus, researches have established conditional transgenic approaches, which could be spatial or temporal control. Spatial control restricts the expression of oncogenes to a specific tissue based on the use of tissue-specific promoters (17). Some methods that allow spatial control are the Gal4/UAS system, site-specific recombinases such as Cre/loxP, Flp/frt, phiC31, and Dre/rox-system (18–22). Temporal control of oncogene expression or inactivation of tumor suppressors can be achieved by heat shock, hormones, Tet-On and Tet-Off system, and optogenetics. Several examples of cancer models carried out in zebrafish using genetics approaches are shown in Supplementary Table S5.

Transplantation of tumor cells in zebrafish

Another approach to generate cancer in zebrafish is the transplantation of tumor cells. Diverse engraftment assays can be carried out in zebrafish (Fig. 1B). This model of cancer induction is an ideal tool to understand the processes of angiogenesis, tumor cell extravasation, migration, and metastasis (23–25). This procedure has many variables to consider, such as the origin of the donor material. Most of the studies are criticized for use of established cell lines to carry out xenotransplantation assays. This is not considered to be presenting the same conditions of a cancer, as it has been proven that the tumor microenvironment changes spatially and temporally (26). In addition, there is heterogeneity in a tumor, as well as genetic evolution that a commercialized cell line cannot offer (27). Another variable is the microinjection site of the tumor cells, which can vary depending on the developmental stage of the zebrafish. Yolk is the most common injection location, as it provides a large site to house transplanted cells and facilitate manual transplantation in comparison with other smaller regions also injected in zebrafish, such as the duct of Cuvier, caudal vain, or heart (26). Cells to be injected require *in vivo* pretreatment with cell membrane stains such as CMDiL or transfection to express GFP for visualization after transplant because of the fluorescent signal (26). At present, it is common to screen embryos injected with the appropriate number of cells, as it is still challenging to obtain a reproducible volume of cell administration (28).

One of the main drawbacks of transplantation is the immune rejection of the inoculated tumor cells. In mouse models, a strategy to avoid that process is the use of the NOD/SCID mouse, which presents multiple immunologic alterations, such as the immunosuppression of T, B, and natural killer cells (29). In addition, some chemicals and irradiation are able to act as suppressors of the immune system (30). Zebrafish embryos have not completely developed their innate and adaptive immune system until 21 days of life (31). At that moment, immature T and B cells reach the thymus, finalizing the process of immune maturation (32). This lack of immune defense until 3 to 4 days post fertilization (dpf) prevents the requirement of immunosuppression, and thus, an embryo model is preferable in transplantation assays (33).

In contrast, adults require immune system ablation to avoid engraftment rejection. An important fact to take into account in zebrafish cancer models is the stage in which the assays are carried out. Methods applied to achieve immunosuppression in adult zebrafish are similar to mouse model approaches. Traver and colleagues proved that sublethal radiation (20–25 Gy) produced immune ablation and 90% survival (34). Subsequently, hematopoiesis is reinitiated 12 days after irradiation, and the marrow is fully restored by 20 days after irradiation, killing engrafted cells. In embryos 6 dpf to 1 month old, 15 Gy of gamma-irradiation can ablate T cells (35). Another strategy for immunosuppression is chemical treatment with dexamethasone. This treatment suppresses T and B cells, allowing solid tumor transplantation (36). The 5-day-old zebrafish embryos could be immunosuppressed with 250 mg/mL of dexamethasone 1 to 3 days before transplant (35). Furthermore, transplant could be carried out in immune-compromised zebrafish, allowing long-term engraftment assays and avoiding preconditioning (37). Some immunocompromised transgenic zebrafish have been developed, such as *recombinant activating gene 1* (rag 1) or *v-myb avian myeloblastosis viral oncogene homolog* (myb) mutants. However, these mutants are not commonly used in transplant experiments because the lines are difficult to maintain, and they have other associated diseases (38, 39). Most recently, a *recombination activating gene 2* (rag 2) mutant has been used to transplant tumor cells (40). The major disadvantage of the immunosuppressant method is the inability to study the relationship between immune cells and tumor growth (37).

The transplantation of tumor cells from a donor fish to a genetically identical recipient, known as clonal or syngeneic fish, avoids the immunosuppression requirement (41). In this case, as the immune system is fully activated, the study of interaction between immune cells and tumor is feasible, as is long-term engraftment. However, this method has the limitation of the complexity in the line achievement (37). Zhang and colleagues developed a novel tumor cell transplantation strategy without immunosuppression requirement. This method consists of transplanting irradiated human tumor cells into a zebrafish embryo and retransplanting nonirradiated cells into the same zebrafish 3 months later (42).

Allotransplantation of zebrafish tumor cells. Allogenic transplantation is the transfer of cells, organs, or tissues from one individual into another of the same species. In allograft assays, donor zebrafish suffering from cancer could be obtained by all the methods previously described. Zebrafish recipients require preconditioning treatment if they are not syngeneic or immunosuppressed individuals (Supplementary Table S6).

Xenotransplantation of tumor cells. Xenografting is the process of implanting living tumor cells from one species to another. Lee and colleagues performed the first xenotransplant of human cancer cells to zebrafish embryos to resemble melanoma (43). Tumor cell behavior in zebrafish xenograft models correlates with human cancers (44). Most of the assays carried out are implemented in zebrafish embryos owing to the advantages that this developmental stage offers (Supplementary Table S7).

However, Stoletov and colleagues transplanted several tumor cell lines, such as fibrosarcoma (HT1080) and melanoma (MDA-435, B16), into juvenile 25- to 30-day-old zebrafish treated with dexamethasone to study metastasis by confocal imaging and histology assays (35).

An emerging approach to translational cancer research is the patient-derived xenograft in zebrafish embryos (zPDX). Tumor cells from primary or metastatic human cancers collected by surgery or biopsy procedures are transplanted into zebrafish. This approach provides information about the effectiveness of a treatment, as cells have the same molecular, genetic, and clinical characteristics as the donor. PDX have been broadly developed in mouse models. However, this model presents some limitations that zPDX overcome such as the time required to develop tumor and sample quantity required from each patient (45). Models of several cancer types have been developed using this technique as gastric, breast, or neuroendocrine cancers (46–48). Furthermore, Fior and colleagues showed the reliability of this model by comparing responses to chemotherapy and biological therapies between patients and colorectal zPDX (49, 50).

Orthotopic transplantation of tumor cells. Another approach to xenograft experiments is the orthotopic transplantation of tumor cells. This consists of cell implantation into the same site or organ in which cancer has developed in the donor. Some orthograft assays performed in zebrafish are shown in Supplementary Table S8.

Zebrafish embryos are transparent, allowing visual observation of labeled tumor cells by imaging equipment. Consequently, embryos are the most common stage selected for this sort of study. However, they have not developed every adult organ yet, limiting the tissues where orthotopic transplantation could be performed. In most of the transplantation experiments, cells are inoculated into the yolk of zebrafish embryos, avoiding an orthograft approach. In other cases, it is not possible due to the absence of a concrete organ in zebrafish, such as for breast, lung, or prostate cancer (26). Therefore, Eden and colleagues successfully transplanted mouse tumor cells into the brain of a 30-day-old adult zebrafish previously immunosuppressed with dexamethasone (51). The orthotopic transplantation is more efficient and closer to human metastasis (52). The main disadvantage of this method is the time-consuming and complex nature of the procedure, as well as the limitation of imaging monitoring.

Monitoring Cancer Processes in Zebrafish

Once the cancer induction or engraftment is accomplished, *in vivo* monitoring of tumor processes in zebrafish requires specific and expensive imaging techniques and qualified personnel. Some of the approaches used in cancer monitoring in zebrafish are shown in Supplementary Table S9.

In terms of transplantation assays, there are some strategies to track and label *in vivo* tumor cells using fluorescence microscopy such as fluorescent protein-based reporters or labeling approaches without the gene transfer requirement. At present, membrane dyes as lipophilic carbocyanine dyes (DiO, DiI, DiD, and DiR) have become routinely used to image real-time cancer process at the single-cell level (53).

Furthermore, many imaging methods have been recently developed to enable more accurate imaging analysis. Ghotra and colleagues established a quantitative bioimaging platform to study human cancer dissemination in a xenograft assay (54). Kumar and colleagues described 3D-fluorescence imaging using angularly multiplexed optical projection tomography with compressive sensing to observe tumor progression and vasculature development in live, nonpigmented adult zebrafish (55). In terms of screening assays, in which researchers need to analyze large numbers of images, Pardo-Martin and colleagues developed a vertebrate automated screening technology (VAST) that allows automatic manipulation and imaging collection (56). Then, this tool was improved as the VAST BioImager system, providing automatic handling, positioning, orientating, and high-resolution imaging collection (57).

As has been previously introduced, zebrafish is a very versatile model, which allows the development of many transgenic individuals, improving the study of cancer processes. White and colleagues described a transparent adult zebrafish called *casper*, which has homozygous mutations in two pigmentation loci (7). Benjamin and Hynes were able to visualize *in vivo* metastasis by using this zebrafish mutant after the ZMEL1 cell transplantation (58). Heilmann and colleagues developed a quantitative system to study metastasis to end up with the semiquantitative detection and low signal-to-noise ratio analyses (59). Chen and colleagues created a transgenic line to facilitate luciferase-based imaging in zebrafish, allowing deep tissue visualization in freely swimming animals (60). Furthermore, some transgenic zebrafish lines have the vasculature marked, such as *fli-GFP*, *mtie2-GFP*, and *flik-EGFP* (61–63).

Tumor cell transplantation methods together with the diverse available imaging tools serve to visualize and clarify the insight of tumor processes. (i) Park and colleagues described pancreatic tumor initiation in a KRASG12V transgenic zebrafish model (64). (ii) Neovascularization and behavior of metastatic adenocarcinoma MD-435 cells were visualized in a *fli2-EGFP* transgenic model (35). (iii) Ghotra and colleagues observed migration and dissemination of prostate CMDil-labeled cells transplanted into a *flik-EGFP* transgenic zebrafish (54). (iv) Invasion assays were carried out by xenografting human tumor cells into the same transgenic zebrafish (65). (v) Tumor and immune system interaction was optically studied by Wang and colleagues when human ovarian cells were transplanted into vascularized-labeled zebrafish (66).

Other *ex vivo* approaches monitor tumor processes in ways that differ from imaging. For instance, xenotransplantation observation could be performed by dissociating injected embryos into a

single-cell suspension and counting the average number of fluorescent cells with a microscope (67). In other studies, qPCR was performed to detect a cancer-specific gene or human housekeeping gene in order to evaluate tumor progression (68, 69).

Zebrafish Cancer Model in Drug Discovery

Because of all the previously described advantages that the zebrafish animal model presents, it has recently stood out in the drug discovery process (i) to identify molecules that specifically ameliorate a disease phenotype and (ii) to perform detailed characterization studies around optimized compounds, focusing not only on efficacy (dose-response) but also on toxicity and/or mechanism. Furthermore, personalized treatments are feasible thanks to the development of zPDX, looking at precision cancer medicine.

In terms of cancer, to the best of our knowledge, there are 355 cases reported in the literature where this animal model became a fundamental tool in the drug discovery process, from the discovery of new compounds, or known drugs, with antitumoral activity to detailed therapeutic assessment of optimized molecules (dose-response, toxicity, and/or pathway studies).

Zebrafish in screening campaigns: Advantages and setup considerations

Small-molecule screens are widely used to identify new therapeutics. In this context, cell-based and biochemical drug screenings have played an important role in the identification of new active molecules from large libraries of compounds. Nevertheless, in recent years, whole organism screenings have emerged as a promising alternative to test thousands of molecules. Zebrafish is undoubtedly an interesting approach for this purpose, representing a reliable, low-cost, and rapid option to perform screenings of large libraries and assess their immediate therapeutic relevance. Rennekamp and Peterson reviewed the advantages in zebrafish chemical screening, its limitations, and the impact of zebrafish on chemical biology (70).

Murphey and Zon broadly reviewed the small-molecule screening methods that could be performed (71). These assays carried out in zebrafish consider *in vivo* small-molecule activity and take into account metabolism, toxicity, pharmacokinetics, pharmacodynamics, and cell–cell interactions, providing important information in an early developmental stage that cannot be obtained with traditional biochemical or cell-based screenings (72). Furthermore, this methodology allows the identification of a therapeutic compound without knowing the exact mechanism of the disease (73). In addition, an advantage against the traditional murine model is the requirement of fewer amounts of experimental chemicals, reducing the difficulty and costs associated with the collection procedures (74).

In terms of logistics, screenings with zebrafish can be adapted from 6- to 384-well plates using a variable number of embryos per well, but assays are commonly conducted in 96-well plates. Until now, distribution of these embryos into plates has mainly been performed by hand, but recently, this process has also been automated (75). This fact, together with the possibility of obtaining large numbers of synchronized embryos, opens the possibility to screen larger compound libraries. Regarding the readout of the screening, a wide variety of scoring phenotypes can be adapted to these screenings, depending on the study goal. Especially interesting are morphology changes that can be easily observed in early stages of life thanks to the transparency of zebrafish larvae. According to assay output, phenotypic screening could be morphologic, therapeutic, pathway, or behavioral (76).

Different libraries of compounds can be used in zebrafish screening, from small collections of characterized compounds to larger libraries of thousands of compounds (77). The election of the drug library applied to the screening test depends on the aim of the study. Novel compounds libraries are applied to identify new chemical series and/or mechanisms of action. The corresponding initial hits may initiate a drug discovery project; on the other hand, testing FDA-approved compounds may lead to drug repositioning.

Compound administration and pharmacokinetics

The classical administration of drug is achieved by dissolving the compound directly in the fish water (8). Zebrafish embryos are able to absorb solubilized compounds, allowing feasible administration (16). Zhang and colleagues showed that 3 dpf zebrafish absorbed drugs through the skin and swallowing (78).

However, this method has associated challenges to overcome, such as the variability in the molecule solubility, possible precipitation, and the permeability of the compound. If the drug is not soluble in water, vehicles such as dimethyl sulfoxide can be used, as zebrafish can survive in solutions of 1% (28). Direct administration requires invasive intraperitoneal or retro-orbital injection, which could prevent long-term drug assays (79, 80). To overcome this drawback, Dang and colleagues developed an oral gavage and anesthesia method in adult zebrafish for cancer preclinical studies (81). Furthermore, artificial oil bodies with phospholipids have been developed in order to obtain noninvasive drug administration (82). Kulkarni and colleagues also reported a novel method for oral administration: inserting a micropipette with a small tip into the mouth and pharynx of adult zebrafish, avoiding the variability when chemicals are added to the aquarium water (83).

In addition, zebrafish recently stood out as a tool to develop and test new drug administration strategies such as nanoparticles (84). Therefore, it is difficult to predict how much drug will be absorbed. Depending on the fish developmental stage, entry sites for small molecules are not the same, and as a consequence, the results of the screening can also be different. In addition to using a whole organism, other aspects such as genetic penetrance, *in vivo* chemical modification, or pharmacokinetics (a critical aspect, elaborated below) can alter the results.

Drugs that target human proteins might have different effects in zebrafish, as they present more than one ortholog to human proteins. However, previous pharmacokinetic studies have demonstrated that zebrafish larvae have the ability to perform phase I and phase II metabolism reactions (85). Drug distribution, metabolism, excretion, and allocation into specific organs are replicated in zebrafish, as they possess a full complement of the major drug-metabolizing cytochrome P45O enzymes presented in humans (86). A similarity between zebrafish and higher vertebrates in terms of blood–brain barrier (BBB) permeability has also been demonstrated (87). Together, these data suggest that this animal model could be an excellent model for studying the pharmacokinetic profile of new drugs, but to date, few examples have been published. Kulkarni and colleagues first described a simple method to study the pharmacokinetics of carbamazepine in adult zebrafish, suggesting

that this animal may be an excellent model for studying oral pharmacokinetic and BBB permeability (83).

Later, Kantae and colleagues published the development of an analytical method based on UPLC and mass spectrometric detection to study paracetamol and its metabolites in zebrafish larvae. They used larvae at 3 dpf and concluded that clearance of paracetamol is lower than in higher vertebrates but correlates well to values in immature individuals, probably due to the immaturity of enzymes in zebrafish at 3 dpf (88). Finally, Zhuo and colleagues reported the study of the pharmacokinetic profile and distribution of tramadol and two metabolites in zebrafish by electrospray ionization-quadrupole-time of flight/mass spectrometry and gas chromatography/mass spectrometry. They compared the results using different doses and administration methods (oral and intramuscular) and validated their method for the analysis of different tissues, such as brain, eyes, muscle, and gills (89).

Case studies

This review compiles the case studies reported so far. To the best of our knowledge, there are 355 in which zebrafish was employed in any step of the cancer drug discovery process. As shown in Fig. 2A, most of these studies used zebrafish as *in vivo* models to evaluate the antitumoral efficacy of lead compounds, detailed efficacy studies together with toxicity and/or mechanistic studies (named "Compound activity testing"). In these terms, zebrafish assays enable structure–activity relationship analyses of newly designed molecules, natural bioactive extracts, and analogs of known antitumoral compounds (90–92). Furthermore, within the cases encompassed in this classification, other studies employed molecules with known effects against cancer to decipher the underlying mechanism of action, to validate new targets or to identify novel pathways (90–94). On the other hand, zebrafish screenings have become recently widespread because they provide a feasible tool to perform high-throughput phenotypic screens (73). In this regard, we have identified several cases in which this animal model was used to perform phenotypic screenings of a large number of molecules to identify new hits for a drug discovery project (named "Compound screening" in Fig. 2A). In these studies, proprietary as well as commercially available libraries, both focused and diverse, have been used (95, 96). Finally, repurposing of approved drugs using zebrafish to discover a potential antitumoral indication has been less frequently described (named "Drug reprofiling" in Fig. 2A). To the best of our knowledge, despite the cost- and time-effective advantages of this approach, only 5 successful cases of repurposing have been reported to date. All details of these 355 studies are described in the Supplementary Information and report for each of the following: (i) the aim of the study (activity test, screening campaign, or drug reprofiling), (ii) subject matter, (iii) cancer type, (iv) assay type (xenograft, angiogenesis, etc.), and (v) corresponding reference.

The first compound identified by zebrafish screening that reached phase I clinical trial was reported in 2013. This molecule, ProHema, was discovered after testing 2,500 compounds. ProHema is derived from Prostaglandin E2 and increases the engraftment of umbilical cord blood stem cells in transplant assays (97, 98). At the present time, two clinical trials using this compound against hematologic malignancies have been completed (identifier: NCT00890500, NCT02354417; ref. 99). Taking into account the potential immediate impact of drug repositioning on patients, we want to highlight some successful cases. In 2010, Wang and colleagues identified Rosuvastatin, a compound approved to treat hypercholesterolemia, atherosclerosis, and cardiovascular diseases, as an antiangiogenesis drug. In a zebrafish chemical screening, this drug suppressed prostate tumor growth by inhibiting endothelial cell function (100). Furthermore, at present a phase II clinical trial has been conducted to evaluate the antitumoral effect of Rosuvastatin to treat rectal cancer (identifier: NCT02569645; ref. 99). White and colleagues performed an antimelanoma screening in zebrafish. In this case, inhibitors of dihydroorotate dehydrogenase such as leflunomide, used as arthritis treatment, showed inhibition of transcriptional elongation of genes related with melanoma growth; these molecules have been tested in phase I/II clinical trials for the treatment of human melanoma in combination with vemurafenib (identifier: NCT01611675; refs. 99, 101). On the other hand, using two complementary screenings, Gutierrez and colleagues identified several drugs effective against T-cell acute lymphoblastic leukemia. Although the origin of their antiproliferative activity is unknown, and it is thought to involve several mechanisms of action, zebrafish screening showed the antitumoral activity of perphenazine (PPZ), an FDA-approved antipsychotic drug (102). To date, there is no cancer clinical trial employing this drug (99). Testing a commercial library of pharmacologically active compounds in a transgenic zebrafish screening, Evason and colleagues identified two antidepressants that suppressed the hepatocellular carcinoma phenotype (amitriptyline and paroxetine) by suppressing the β-catenin pathway (103). Furthermore, Fernandez del Ama and colleagues discovered the antimelanoma effects of rapamycin, disulfiram, and tanshinone in synergy with MEK and PI3K/mTOR pathway inhibitors (104). Another successful case of cancer reprofiling in a zebrafish model was the antiangiogenic effect showed by closantel, a veterinary anthelmintic drug (105).

As shown in Fig. 2B, most of the reported studies employed zebrafish (i) to assess the antitumoral activity of compounds by using transgenic zebrafish, phenotype assays, or xenograft experiments, as well as (ii) to resemble a specific human cancer type (named "Specific antitumoral activity"). Furthermore, most of the reported studies test antitumoral effects against more than one cancer type. Many cancer models have been described in zebrafish (16). As shown in the figure, the cancer types most commonly studied in zebrafish in the drug discovery process are breast cancer, leukemia, lung cancer, and melanoma, demonstrating the outstanding versatility of this animal model. As angiogenesis is a crucial process involved in tumor progression and spreading, many anticancer therapies focus on targeting these molecular pathways. Furthermore, lymphatic vessel formation is involved in cancer metastasis and progression, becoming a new target for anticancer therapy (106). The transparency of zebrafish embryos and the development of transgenic zebrafish with labeled vasculature enable the visualization of *de novo* blood and lymphatic vessel formation, providing a feasible zebrafish phenotypic observation to identify hits that disrupt these pathways (studies named "Anti-angiogenic activity" and "Anti-lymphatic activity"; ref. 107). Moreover, signaling pathways involved in embryonic development, such as TGFβ, Notch, or Wnt, as well as cellular mechanisms such as apoptosis and cell-cycle regulation, are also related to cancer development when deregulated (108, 109). In zebrafish, alterations of these pathways can be easily observed

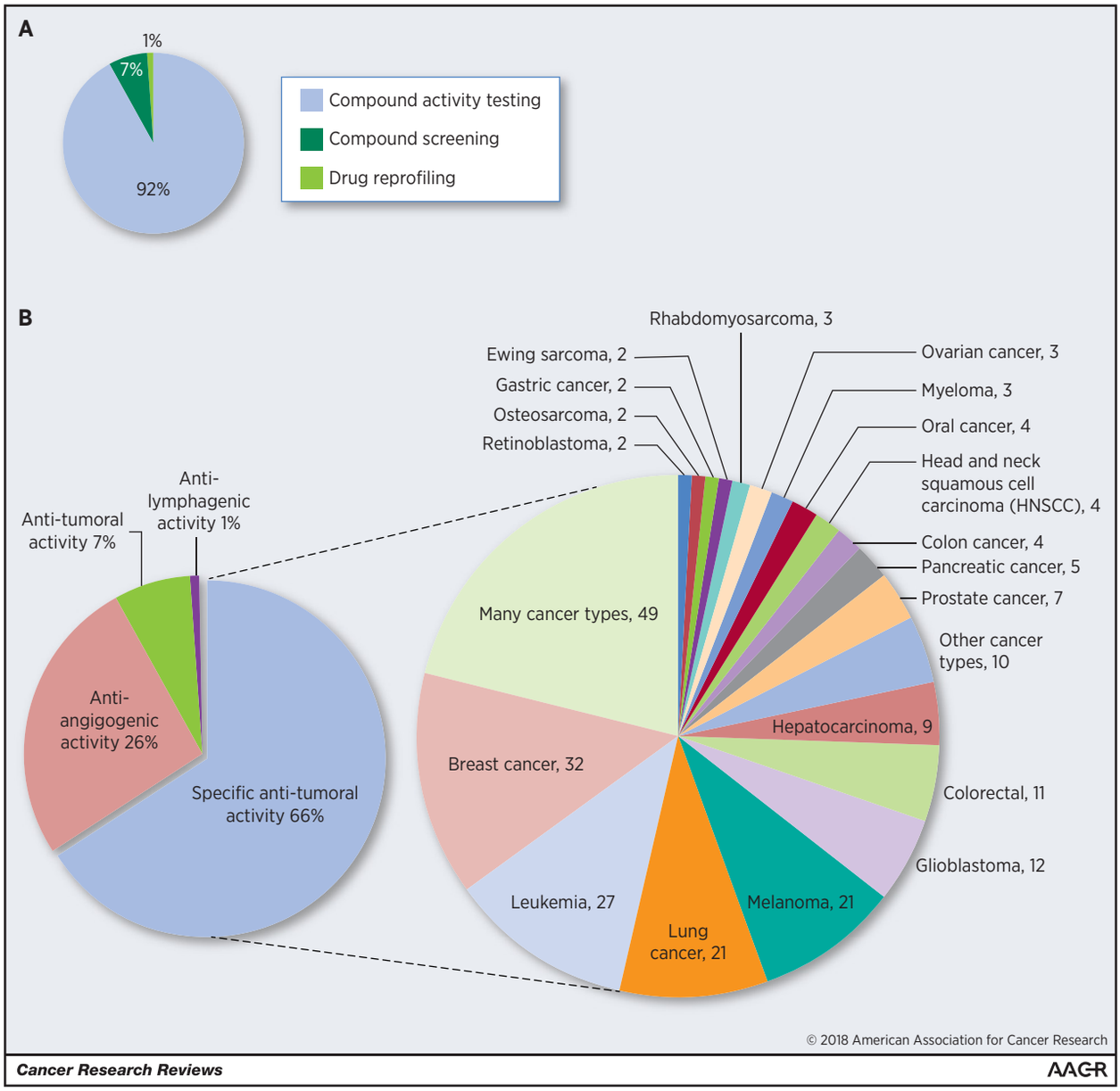

Figure 2.

A, Classification of the 355 reported case studies in zebrafish, cancer drug discovery projects, according to the aim of the study. **B,** Left graph represents studies reported in literature classified by the subject matter. Right graph shows cancer types studied in cases encompass in "Specific anti-tumoral activity." All details about the 355 case studies are described in Supplementary Table S10.

phenotypically as developmental disruptions. Other case studies identified compounds using mutant or transgenic zebrafish to identify antitumoral activity, but not against a specific tumor type (named "Anti-tumoral activity").

Conclusion and Perspectives

The zebrafish *in vivo* model provides many advantages in cancer research in comparison with the broadly used traditional *in vitro* cell model and the *in vivo* murine model. Due to its maintenance costs, work feasibility, and simplicity to obtain cancer phenotypes, zebrafish has recently become a meaningful tool in science. In terms of cancer research, zebrafish allows scientists to study procedures such as tumor formation, migration, and metastasis as well as to perform an agile identification of the optimal molecule, or/and known drug (repositioning), to treat each different tumor types. Some of these strengths are as follows: (i) Adult zebrafish spawns large numbers of embryos in each clutch, providing a high-confidence statistical analysis method (4). (ii) Currently, several approaches to induce cancer in zebrafish have been

broadly described, from mutation and transgenesis to transplantation techniques. (iii) Tumor formation is a rapid process, only requiring from 10–14 days to 1 month, compared with that of the traditional murine model that needs up to 4 months to observe the complete process (12, 110). (iv) Zebrafish cancer has been proven to be similar to human cancer (10). (v) In terms of logistics, embryos can be placed in 96-well plates, allowing high-throughput studies to test thousands of chemical compounds, thereby reducing time and costs in chemical screenings and leading to results of immediate therapeutic relevance (111). (vi) Transparency of the embryos and the development of new mutants without pigmentation, such as the *casper* zebrafish, offer the possibility to visualize all of these cancer processes (7). (vii) Most of the compounds can be dissolved in water as a feasible method of administration (8). (viii) Although there are few cases reported in the literature in which zebrafish was successfully employed in drug discovery and reprofiling, several compounds reached a phase II clinical trial, showing the advantages that the zebrafish animal model provides (99, 100). (ix) Several studies show the reliability of zPDX model for different cancer types as it is able to overcome some drawbacks of the murine PDX such as time required to develop a model ready for preclinical study (45–50).

However, from cancer drug discovery perspective, zebrafish still presents some limitations that should be overcome in a near future. As reported below, there are specific challenges that have to be faced more efficiently; in fact, they require further development and refinement:

- High-resolution imaging techniques. Monitoring cancer in zebrafish needs specific equipment and transgenic animals. In addition, cells should be labeled (7, 112), although new imaging systems have been developed such as a linear-CCD "charge-coupled device"–based flow imaging system that allows high-throughput imaging of dozens of embryos per second (113). Furthermore, real-time zebrafish monitoring has been achieved thanks to the improvement of immobilization techniques. Most of them are based on microfluidic plates and chips that enable cost-effective phenotype-based screenings and feasible drug administration (114, 115).
- Immune system. The majority of studies must be carried out in an embryo stage, as the immune system is not completely developed, and the assays performed in an advanced stage should be immunosuppressed (34, 35). By the application of the techniques described in an advanced development stage, the results would resemble the real behavior of cancer in humans (25). Casey and colleagues achieved the allotransplantation of pediatric brain tumors into immune-competent zebrafish (116). Furthermore, this *in vivo* model has broadened its application in drug discovery processes as it has been recently employed in immunotherapeutic drug screenings (117).
- Compound administration. Direct and long-term administration is a drawback in zebrafish assays, as methods used at present are very invasive, and it is difficult to predict whether the drug was absorbed by the zebrafish (26). Monstad-Rios and colleagues developed a 3D printed system for cost-effective drug administration in adult zebrafish enabling small-molecular screenings in postembryonic models (118).
- Pharmacokinetics and pharmacodynamics. Although it has been demonstrated that larvae have the ability to perform metabolism reactions, and their drug distribution, metabolism, excretion, and allocation are similar to humans, these fields are scarcely explored in zebrafish (86). A very recent study, using a zebrafish orthotopic glioblastoma xenograft model, was able to monitor compounds crossing the BBB and identify a drug that efficiently passes through the BBB (119).
- Tumor microenvironment. Some cancer processes proven to be very relevant in tumor formation, such as the tumor microenvironment, are seldom studied in zebrafish (120). However, a cancer stem cell xenograft model developed by Chen and colleagues enabled the study of interaction with microenvironment during bone metastasis progression (121).

On the other hand, zebrafish has become a versatile and reliable tool in cancer research due to emerging approaches that may have a huge impact on cancer drug discovery process in the near future:

- Bioenergetic-based screening. Ibjazehiebo and colleagues performed an *in vivo* bioenergetic screening in zebrafish for epilepsy (122). As cancer energy metabolism plays a key role in cell progression, tumor bioenergetics stands out as a new target for cancer therapies (123). Therefore, this approach could be currently applied in antitumoral screenings using zebrafish.
- zPDX. This approach is probably the most relevant, from a translational perspective, in cancer drug discovery using zebrafish. Every patient and its corresponding tumor type may respond in a different manner to drugs; then, efficient models providing fast and reliable assessments for personalized treatments might provide an outstanding added value for precision cancer medicine (49, 124).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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