Review

Clinical Cancer Research

Zebrafish: A New Companion for Translational Research in Oncology

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Abstract

In an era of high-throughput "omic" technologies, the unprecedented amount of data that can be generated presents a significant opportunity but simultaneously an even greater challenge for oncologists trying to provide personalized treatment. Classically, preclinical testing of new targets and identification of active compounds against those targets have entailed the extensive use of established human cell lines, as well as genetically modified mouse tumor models. Patient-derived xenografts in zebrafish may in the near future provide a platform for selecting an appropriate personalized therapy and together with zebrafish transgenic tumor models represent an alternative vehicle for drug development. The zebrafish is readily genetically modified. The transparency of zebrafish embryos and the recent development of pigment-deficient zebrafish afford researchers the valuable capacity to observe directly cancer formation and progression in a live vertebrate host. The zebrafish is amenable to transplantation assays that test the serial passage of fluorescently labeled tumor cells as well as their capacity to disseminate and/or metastasize. Progress achieved to date in genetic engineering and xenotransplantation will establish the zebrafish as one of the most versatile animal models for cancer research. A model organism that can be used in transgenesis, transplantation assays, single-cell functional assays, and *in vivo* imaging studies make zebrafish a natural companion for mice in translational oncology research. *Clin Cancer Res; 21(5); 969–75. ©2015 AACR.*

Introduction

In an era of high-throughput "omic" technologies, the unprecedented amount of data that can be generated presents a significant challenge for oncologists trying to provide their patients with the most suitable treatment. On the other hand, the wealth of genetic and transcriptomic data, accumulated through international cancer efforts such as The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium (ICGC), provide numerous opportunities for identifying new therapeutic targets.

Classically, preclinical testing of new targets and identification of compounds active against those targets has entailed the extensive use of established human cell line models. One of the biggest and better described repository of cell lines is the NCI-60 panel (1, 2). However, the scope of this approach for generating clinical leads is limited. This is mainly due to the adaptations that cell lines undergo during the process of establishment in culture, which tend to suppress heterogeneity and compensate for a loss of stromal support. Typically, by the end of the establishment process, we are more than likely to have isolated a highly proliferative clone of cells from the original tumor, which ironically may not represent the most clinically challenging tumor population to treat (3). The problem of bias in selecting clones can be overcome if a spectrum of established cell lines is available, themselves reflecting a spectrum of tumor stages. This is the case for more common tumors such as colorectal neoplasms. But when studying rarer tumors, mirroring the heterogeneity of these diseases *in vitro* becomes an almost impossible mission. To illustrate this problem, while 21 cell lines derived from pancreatic adenocarcinoma are available from the ATCC, no cell lines are available derived from pancreatic neuroendocrine tumors.

To tackle the lack of representative *in vitro* models for target validation and drug screening, researchers have generated more complicated preclinical models. Genetically engineered organisms represent an attempt to recapitulate the complex multistep process resulting in tumor formation and malignant progression. The mouse has become the organism of choice for genetic modification, and recombinant mouse models account for the vast majority of preclinical animal studies in the PubMed database, with other species, such as zebrafish, making a much rarer and more recent appearance (Fig. 1). However, genetic models, while mimicking human disease closely, can be challenging to incorporate into drug development pipelines, owing to heterogeneity in tumor growth and spread as well as challenges entailed in imaging tumors beneath the skin.

An alternative tumor model recently gaining traction is patient-derived xenografts (PDX), whereby tumor cells or tumor fragments freshly isolated from patients undergoing biopsy or resection are implanted in an animal host, again typically a mouse. This technique is not new; it was established in the 1980s (4). The recent popularity of this approach is more reflective of the changing field of oncology and the need to match experimental drugs with a biomarker in an attempt to reduce the costly attrition of clinical candidates in "all-comers" phase III trials. Both industry and academia are working to find a more comprehensive preclinical model that will guide drug development in oncology so that fewer clinical trials fail than at present (5).

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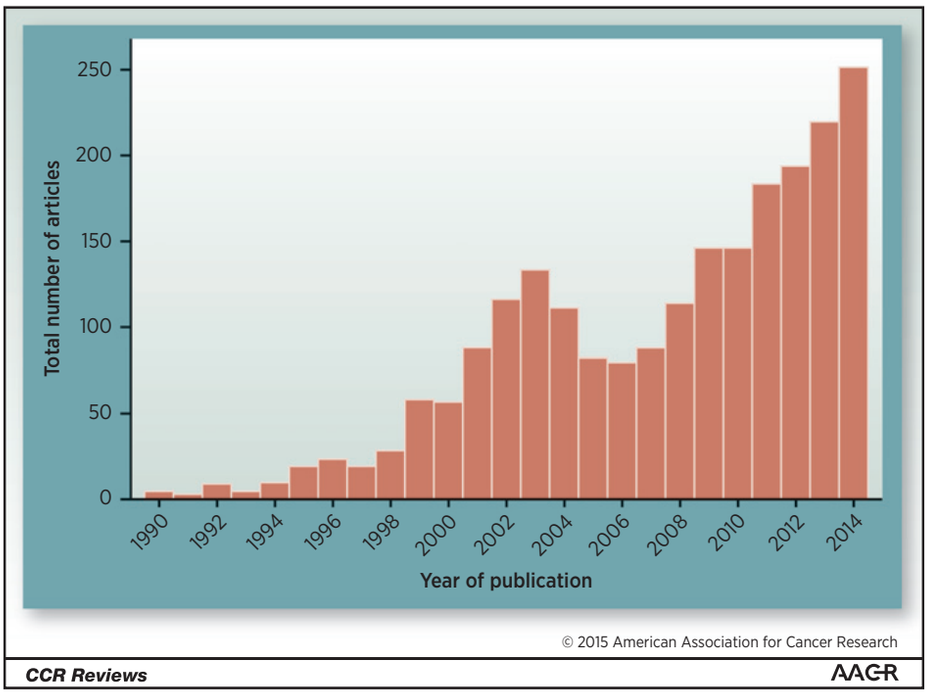

Figure 1.
Absolute number of articles for zebrafish cancer models per year of publication extracted from the PubMed database (61; last accessed on November 1, 2014).

Zebrafish Can Provide Preclinical Tumor Models

The zebrafish has recently emerged as a versatile model system for the study of human cancers (6–9). The zebrafish, a small freshwater tropical fish found indigenously throughout streams and waterways in Northeast India, was first used as a model organism for developmental genetics in the 1960s and was described by pioneer George Streisinger as a "phage with a backbone" (9). Distinct advantages of the fish arise from the evolutionary conservation of genetic pathways implicated in cancer that are shared between fish and humans coupled to the unique attributes of zebrafish as a tool for modeling human disease and analyzing the underlying cellular processes (10). The first experimental approaches included the use of carcinogens (11–14). The transparency of zebrafish embryos and the recent development of the pigment deficient "Casper" zebrafish line afford researchers the priceless capacity for direct observation of cancer formation and progression in the living animal (15). The zebrafish is experimentally amenable to transplantation assays that test the serial passage of fluorescently labeled tumor cells as well as their capacity to disseminate and/or metastasize (7, 16–19). Several research groups have also applied xenotransplantation methods to zebrafish, including material derived from patients, for the study of human cancer cell behavior, encompassing response to therapy, within the context of the whole organism (20–23). The experimental repertoire of the zebrafish allows new lines of inquiry into the *in vivo* processes involved in the pathogenesis of malignancy. Moreover, PDX generated in zebrafish may in the near future provide a platform for selecting an appropriate personalized therapy. In the remainder of this short review, we outline possible applications of the zebrafish to cancer research (see overview in Fig. 2), highlighting its potential as new companions to mice in translational oncology laboratories.

Genetic Recombinant and Transgenic Lines

The zebrafish genome project revealed sequence conservation of myriad genes and identified zebrafish orthologs for 82% of human disease genes (10). The availability of the genome sequence ushered in a new era for zebrafish cancer models allowing the development of both genetic recombinant and transgenic lines with targeted mutations in oncogenes and tumor suppressor genes, beginning in the previous decade with the development of melanoma and leukemia models (24, 25). A zebrafish model of melanoma created at the Zon laboratory by Patton and colleagues first confirmed the capacity of $BRAF^{V600E}$ to initiate nevi and melanoma formation (24). The expression of $BRAF^{V600E}$ under the control of the micro-opthalmia–associated transcription factor (*mitf*) promoter caused the formation of nevi in zebrafish. When this construct was injected on a p53 loss-of-function (LOF) background—a technique called TILLING (targeting induced local lesions in genomes) was applied to find mutations in *tp53* after treating zebrafish with ethylnitrosurea (12)—the outcome was the development of melanoma, also providing the first confirmation that p53 inactivation drives progression of melanocyte neoplasia. Subsequently, Iyengar and colleagues from the Zon laboratory engineered a specialized transposon-based vector called "MiniCoopR" that combines a wild-type copy of the *mitf* melanocyte specification factor with a Gateway recombination cassette into which candidate melanoma genes can be recombined (26). When this construct is injected into zygotes from triple mutant Tg (*mitf:* $BRAF^{V600E}$); $p53^{LOF}$; $mitf^{-/-}$ zebrafish, the rescued melanocytes are prone to develop into melanoma, which can be further augmented by the candidate melanoma gene included in the vector. This system is amenable to screen candidate genes derived from any other approach such as next-generation sequencing of recurrent melanoma cases for their ability to cooperate with $BRAF^{V600E}$ in melanoma development.

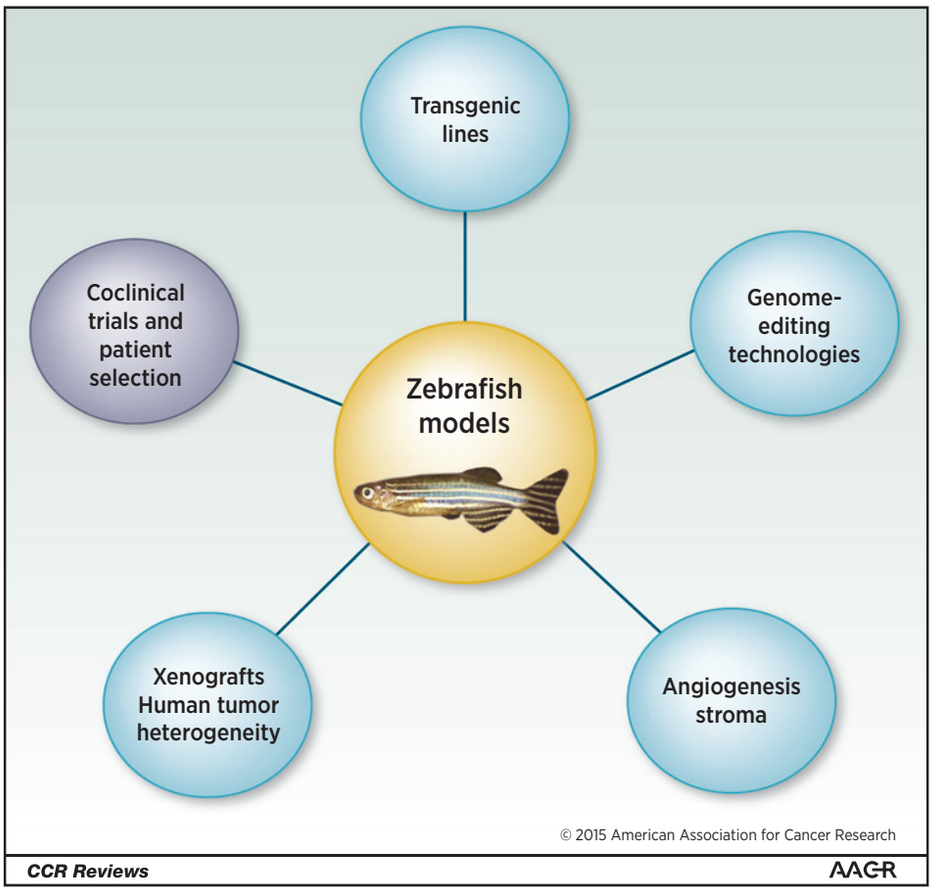

Figure 2.
Roles of zebrafish tumor models in present and future cancer research.

Through this approach, the oncogenic activity of the chromatin-modifying enzyme SETDB1 was first established (26). Numerous hematologic and solid tumor models have now been generated in zebrafish, largely through transgenesis, as have recently been reviewed (6).

Genome-Editing Technologies

Recent technological developments now allow for efficient and pinpoint mutation of genomes (genome or gene editing) of various organisms including the zebrafish, which is set to dramatically expand the repertoire of cancer-associated gene alterations that can be evaluated in zebrafish. Transcription activator-like effectors (TALE) were originally discovered as part of the host–pathogen interaction repertoire in plant cells (27). A pair of two TALE-nuclease (TALEN) fusion proteins is employed to target a specific genomic locus. Each TALEN half comprises a fusion between the nonspecific cleavage domain from the naturally occurring Type IIS FokI endonuclease and a TALE DNA recognition domain (28). Upon binding of two TALENs to their respective target sites separated by 10–20 bases, dimerization of FokI subdomains reconstitutes an active nuclease domain. This leads to cleavage of the targeted genomic locus by inducing a double strand break. Repair of the break invariably introduces small insertions or deletions (indels) that mutate the target site (29).

The CRISPR/Cas (clustered regularly interspaced palindromic repeats/CRISPR-associated) system provides bacteria with a defense mechanism against invasion of foreign nucleic acids. It is a three-component system formed by an array of small CRISPR RNAs (crRNA) encoded in part by the invading foreign DNA, auxiliary *trans*-activating crRNA (tracrRNA), which hybridizes to the crRNA and a nuclease associated with the CRISPR locus (so-called Cas; ref. 30) that forms a complex with the tracrRNA/crRNAs duo. Through complementary base-pairing the CRISPr/Cas complex binds to foreign DNA and introduces a double strand break. Small guide (sg) RNAs are synthetic hybrid RNAs fusing crRNAs with tracrRNA. For gene editing, it is necessary only to replace the 20 bp of the sgRNA responsible for target recognition with a target sequence of interest. After transcription of the customized sgRNA, it binds to its complementary locus in the genome and directs Cas9 to this target site, resulting in target site cleavage and resultant generation of indels (31).

Both TALENs and CRISPR/cas9 technologies have been rapidly adopted by the zebrafish research community. CRISPR/cas9 technology has advantages against TALEN technology in terms of the cloning required to prepare the plasmid for mRNA synthesis, the ability of targeting several loci at the same time and generating a phenotype already in the first generation of injected animals (32). The first report of the use of CRISPR/Cas9 system in zebrafish showed how five genomic loci could be disrupted simultaneously (33). Furthermore, efficient strategies have been devised of combining gene-editing (through TALENs or CRISP/Cas) with homologous recombination (29) to "knock-in" exogenous DNA such as loxP recombination sites to be combined with Cre-mediated

Table 1. Injection sites used for engrafting human cell lines in zebrafish embryos 48 hours postfertilization

Injection site	Cancer type	Cell line name	Reference
Hindbrain ventricle	Melanoma	WM-266-4	(40)
Hindbrain ventricle	Colorectal	SW620	(40)
Duct of Cuvier	Breast	MDA-MB-231	(42)
Caudal vein	Leukemia	Kt562	(54)
Caudal vein	Leukemia	Jurkat	(54)
Caudal vein	Leukemia	NB-4	(54)
Yolk sac	Breast	MDA-MB-231	(43)
Yolk sac	Melanoma	WM-266-4	(40)
Yolk sac	Neuroblastoma	U87-L	(45)
Yolk sac	Leukemia	K562	(23)
Yolk sac	Leukemia	Jurkat	(23)
Yolk sac	Leukemia	NB-4	(23)
Yolk sac	Ovarian	OVCA-433	(44)
Yolk sac	Pancreatic	PaTu	(20)
Yolk sac	Pancreatic	Panc-1	(20)
Yolk sac	Prostate	PC3	(46)
Yolk sac	Sarcoma	U20S	(7)
Yolk sac	Sarcoma	TC32	(7)
Perivitelline space	Breast	MDA-MB435	(39)
Perivitelline space	Sarcoma	TC32	(59)
Perivitelline space	Sarcoma	CADO-ES	(59)
Perivitelline space	Sarcoma	EW3	(59)
Perivitelline space	Sarcoma	EW7	(59)
Perivitelline space	Sarcoma	L1062	(59)
Perivitelline space	Sarcoma	SK-N-MC	(59)
Perivitelline space	Sarcoma	TC71	(59)
Perivitelline space	Thyroid	TT	(60)
Perivitelline space	Lung	DMS79	(60)
Pericardial cavity	Melanoma	501mel	(16, 17)
Pericardial cavity	Melanoma	A375	(16)
Pericardial cavity	Melanoma	WM-266-4	(17)
Pericardial cavity	Melanoma	UACC62	(17)
Pericardial cavity	Melanoma	888mel	(17)

recombination, or cDNA encoding GFP for the creation of fluorescent reporter animals. With this useful addition, it should in time be possible to create conditional alleles that are recombined in specific cell lineages, a technological barrier which until now has distinguished mice from zebrafish.

Xenograft Models and Human Tumor Heterogeneity

The most established approach to model human malignancies in zebrafish is transgenesis, but recently the xenotransplantation of human cell lines has been successfully performed. Xenotransplantation involves the transfer of one species-specific tissue to another animal species (34, 35). Using embryos for this approach has the advantage that the adaptive immune response is not completely developed up to the end of the first month (36, 37), preventing rejection of the graft. Since its trial by Lee and colleagues (38), the technique of engrafting human cancer cells in zebrafish embryos has evolved. The most important parameters in terms of engraftment success are the number of cells injected and the incubation temperature. The temperature is a critical factor, as fish larvae develop better at 28°C while human cancer cells are adapted to grow at 37°C. Several groups report 34–35°C as a compromise allowing growth of both the xenograft and host (16, 39–41). Investigators have also experimented with different anatomical sites for injection, with the yolk sac appearing the one most preferred. The choice of the yolk sac possibly reflects its capacity to contain cells while its function appears not to be compromised.

The different cell lines so far tested in xenotransplantation (Table 1) are melanoma (16, 17, 40), colorectal cancer (40), breast cancer (42, 43), leukemia (23), ovarian cancer (44), neuroblastoma (45), pancreatic cancer (20, 21), prostate cancer (46), and sarcoma (7). Typically, cells are dye labeled to allow their identification within the living host and their growth and infiltration of host tissues monitored over 2 to 5 days. Treatment of engrafted embryos with drugs can result in graft stasis or regression, mirroring outcomes that can be observed in more costly and lengthier mouse xenograft experiments (16). Coinjecting two distinct melanoma cell lines that mimicked the heterogeneity of human melanoma tumors into the pericardial space of zebrafish embryos, we recently demonstrated that melanoma clones cooperate to invade, with certain tumor subsets specializing in leading the infiltration while other subsets specialize in coordinating efforts such that the ensemble move forward collectively (17). We have also used a zebrafish embryo xenograft model to demonstrate the role of the E3 ubiquitin ligase HUWE1 in promoting dissemination of human lung cancer cells (47). In our laboratory, we transplant cells into the thorax (pericardial cavity) of the embryo. Dissemination of cancer cells outside of the thorax results from local invasion.

Recently, the first orthotopic model of mouse brain tumors in adult zebrafish was reported (18). Initially, brain tumor cells were collected from mice and cultured with the temperature being reduced by 0.75°C per week for 4 weeks to adapt growth to 34°C. Meanwhile, wild-type or *Fli1:eGFP* transgenic adult zebrafish were acclimatized to an environmental temperature of 34°C and treated with dexamethasone (15 mg/mL) to suppress adaptive

immunity. Finally, the mice brain tumor cells were injected into a cerebral hemisphere. The investigators showed how the resultant zebrafish orthotopic model recapitulated the histology of their parent tumor and how spread of the tumor cells could now be live imaged, which was not possible in the original mouse host. This study exemplifies how a zebrafish model can complement a mouse tumor model.

Angiogenesis

The models developed to investigate tumor angiogenesis *in vivo* represent another successful application of xenotransplantation in zebrafish. The imaging advantages of the zebrafish embryo have been further enhanced by the development of new techniques and tools for vascular imaging (48). These include a confocal microangiography technique for visualizing patent blood vessels, a complete anatomical description of the early vasculature of the zebrafish, generation of transgenic fish with fluorescently "tagged" vessels, and formulation of methods for long-term video time-lapse microscopy (49–53). The small size of zebrafish embryos also allows them to receive sufficient oxygen for the first few days of development by passive diffusion alone, allowing other organs and tissues to continue to develop normally for several days in the absence of a functional cardiovascular system and greatly facilitating analysis of the specificity of vascular manipulation. Researchers have been able to combine genetically modified cancer models with fluorescently labeled vessels to visualize tumor neovascularization in exquisite detail and also to test antiangiogenic therapies (39), which remain a field of drug development lacking biomarkers in the clinic.

Future Perspectives

The technique of xenotransplantation into zebrafish can also be used with patient-derived tissue, and this has been demonstrated previously for pancreatic adenocarcinoma, prostate cancer, and leukemia (20, 54, 55). The total sample needed for this approach could be as little as 100 cells. The time for engraftment was between 2 and 3 days. Given these characteristics, PDX in zebrafish could be used as a predictive tool for patient responses to drug treatments. The valuable biopsy tissue from one of our patients could be injected into scores of zebrafish embryos potentially with different reporter constructs in the background and different treatments applied to select the most suitable approach. However, more proof-of-principle studies are needed to fully evaluate the value of PDX in zebrafish.

Mouse PDX compared with their zebrafish counterpart have the advantage of offering a well-established model used extensively by academia and the drug industry. This has been translated into the existence of repositories of PDX for different cancers that are commercially available. Tumor grafts in mice are amenable to reliable pharmacokinetic assays, offering the opportunity of mimicking pharmacodynamic/pharmacokinetic relations as performed in early clinical drug development. Mouse PDX are already used in coclinical trials (56, 57).

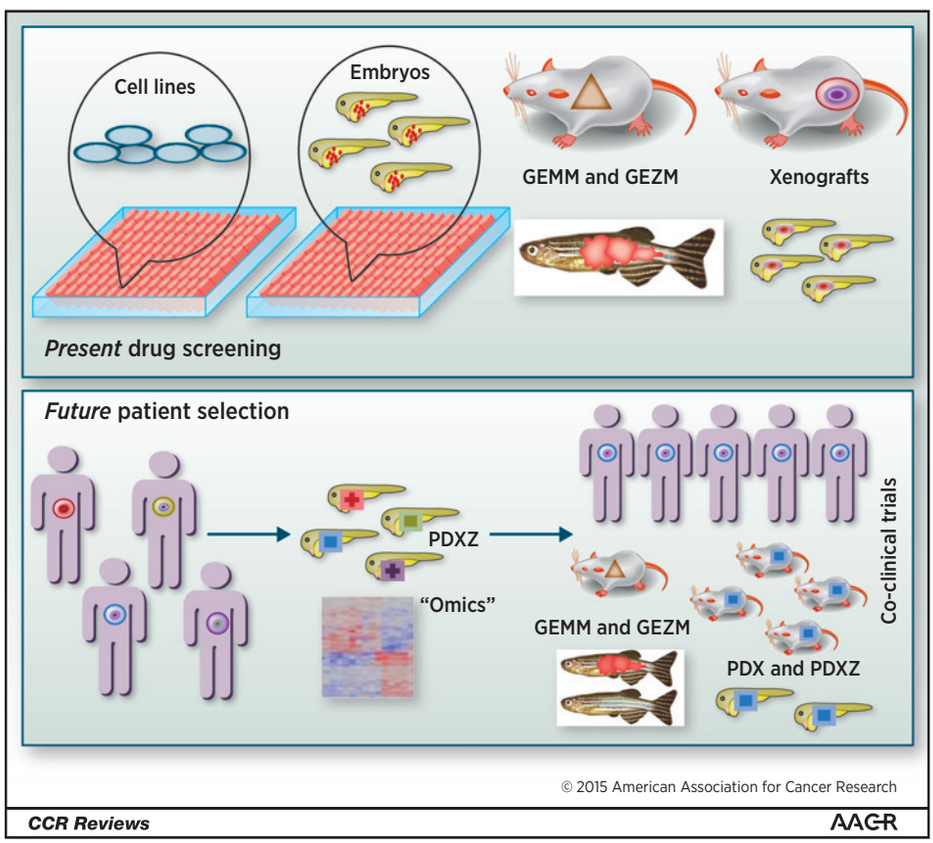

Figure 3.
Potential of zebrafish tumor models in coclinical studies. Zebrafish combine the characteristics of cell lines and animal models and therefore genetically engineered zebrafish models (GEZM) and xenografts are currently used for drug screening and preclinical drug development. In the future, tumor grafts inserted in zebrafish embryos and mice could be used for patient selection in clinical trials. A GEZM could be used to fill the gap whenever a mouse model does not exist. GEMM, genetic engineered mouse models; PDXZ, patient-derived xenografts in zebrafish embryos.

Remaining issues concerning the use of zebrafish include whether the engrafting process leads to changes in tumor cell phenotypes that do not reflect cell behavior in the original lesion; the difficulty to maintain grafts using a multipassage technique similar to PDX in mice; and also the absence of certain organs in the fish (lungs, mammary glands, and prostate) that may preclude studying the tissue-specific mechanisms of homing and colonization by cancer cells. The absence of established pharmacokinetic assays is one of the main pitfalls using zebrafish as host in drug development. Some groups are pioneering approaches to overcome this difficulty, including administering drugs directly to the digestive tract in adult zebrafish (58). All the aforementioned unknowns are related to the early stage of development of this technology.

Conclusions

The zebrafish is a versatile model for the study of cancer. A model that can be used in transgenesis, transplantation assays, single-cell functional assays, and *in vivo* imaging studies makes the zebrafish a natural companion for mice for translational oncology researchers. Zebrafish PDX could be used to provide initial data rapidly in coclinical trials, whereas mouse models need longer incubation times to provide data (see Fig. 3). In the near future, zebrafish PDX could provide meaningful and almost real-time data used to select drugs for patient treatment. Furthermore, whenever the genomic alterations of a given tumor have no direct translation into drug targeting (such as mutations in tumor suppressor genes) an empiric approach based on responses to drugs of zebrafish embryo xenografts could be a valid strategy. The future of zebrafish models envisages them as the perfect companion for mouse assays. Combining the inherent advantages of both models, a more comprehensive use of the data generated by next-generation sequencing studies and the future high-throughput proteomic platforms could be achieved. In summary, the zebrafish offers a very versatile system that could potentially furnish clinical data rapidly and open up new avenues in translational research in a more cost-effective and timely manner.

Disclosure of Potential Conflicts of Interest

A. Hurlstone reports receiving a commercial research grant from MedImmune. No potential conflicts of interest were disclosed by the other authors.

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